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2- and 4-Hydroxyalkyl estradiols were synthesized as biochemically stable Catechol estrogen mimics. These analogs were used as chemicalprobes to evaluate the proposed involvement of catechol estrogens in the initiation of breast cancer. Potentiometric studies performed in PBS buffer at pH 7.4 showed that catechol estrogens were able to undergo redox cycling whereas the Hydroxyalkyl estradiols unable to redox cycle. In agreement with potentiometric studies we have shown that Catechol estrogens are able to induce 8-oxo-dG formation in calf thymus DNA. Our studies have shown that presence of Cu(II) causes a significant increase in DNA damage induced by Catechol estrogens. The Hydroxyalkyl analogs failed to induce 8-oxo-dG formation even in presence of Cu(II). It is important to note that the Catechol estrogens can produce DNA damage at concentrations much higher than their normal physiological levels.

Estrogen A-ring fused heterocycles were synthesized as potential inhibitors of Estrogen hydroxylase, the enzyme responsible for producing Catechol estrogens in the body. 2-Methoxymethyl estradiol was identified as an inhibitor of tubulin polymerization.

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Special Note

Annual report dated July 1997 described the synthesis and preliminary biochemical evaluation of catechol estrogen analogs. *A manuscript describing this work was published in Journal of Medicinal Chemistry*. Reprint of this manuscript has been included with this report.

This report describes a detailed study of measurement of oxidative DNA damage by catechol estrogen analogs as proposed in the statement of work. This work is in collaboration with another graduate student, Mr. James Mobley, in Dr. Brueggemeier's research group. *A manuscript resulting from this work is presently under review for publication in Chemical Research in Toxicology.*

Additionally, novel synthetic approaches for constructing benzopyrone combinatorial libraries were investigated. This work, not described in the original research proposal, describes new developments in this project and aims to identify novel small molecules useful in treatment of breast cancer.

Body is divided into two parts:

Part A: Measurement of oxidative DNA damage by Catechol Estrogen Analogs

Part B: Chemistry for synthesis of estrogen A-ring fused heterocycles, alkoxyalkyl estradiols and synthetic approaches for benzopyrone combinatorial libraries

References for both the parts are arranged separately.

General Introduction

Catechol estrogens (2- and 4- Hydroxy estradiols, HE_2's) are estrogen metabolites formed by the action of specific NADPH CYP-450 hydroxylases. Enzymes responsible for Catechol estrogen formation are predominantly found liver, however smaller levels of these enzymes are expressed in extra-hepatic organs like mammary glands, uterus, brain and other target tissues for estrogen action. There is growing evidence, which indicates that catechol estrogens exert biological actions of their own by interacting with the estrogen receptor or by activating their own unique receptors or effectors. Catechol estrogens are also believed to be the initiators of estrogen dependent tumors.

We are interested in studying the biochemical role of catechol estrogens and modulating the enzymes responsible for catechol estrogen formation. Catechol estrogens are chemically unstable and thus their use in *in vitro* and *in vivo* studies is very difficult. Our goal was to synthesize stable catechol estrogen analogs that could be used to study the biochemical functions of catechol estrogens and shed light on their putative involvement in estrogen induced tumors. To, this end we have synthesized a series of 2- and 4-hydroxyalkyl estrogens as biochemically stable catechol estrogen mimics. These compounds have two oxygens on the A-ring required for interacting with receptors and enzymes. However because of an alkyl spacer between one of the oxygen at 2- or 4-position and the A-ring they are unable to undergo redox cycling and therefore are

chemically more stable than catechol estrogens. These analogs are similar to catechol estrogens in whole cell ER binding assay and in pS2 gene expression studies.

We compared our redox stable analogs with catechol estrogens in potentiometric and DNA damage studies. The potentiometric measurements indicated that both the 2-and 4- HE2's are chemically reversible with half wave potentials of 263 and 265mV±10mV respectively in PBS buffer at pH 7.4. The hydroxyalkyl estrogens were irreversibly oxidized at around 600mV Vs NHE. Inorder to evaluate the tumorigenic potential of catechol estrogens we exposed calf thymus DNA to varying amounts of catechol estrogens and their non redox cycling counterparts and measured the elevated levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG) as marker of oxidative DNA damage. The DNA was analyzed at specific time intervals using reverse phase HPLC system coupled with an electrochemical detector. 8-oxo-dG formation was significantly increased by CE's . Addition of CuSO4 caused a substantial increase in 8-oxo-dG for both CE's. However, a minimum of 10uM of CE was required in order to induce formation of 8-oxo-dG levels higher than the baseline. The hydroxyalkyl analogs did not increase 8-oxo-dG formation even in presence of CuSO4. Reactive Oxygen Species (ROS) scavengers were used to identify H_2O_2 and $^{\bullet}O_2^-$ as the primary oxygen species produced in this system. The effect of lactoperoxidase (LP) on 8-oxo-dG induction by CE's was studied. LP was found to catalyze peroxide driven oxidation of both CE's in a manner consistent with $2e^-$ transfer mechanism, causing a decrease in 8-oxo-dG over controls containing either peroxide or CE alone.

In addition to the above studies estrogen A-ring fused heterocyclic analogs were synthesized as potentially selective inhibitors of estrogen 2- and 4-hydroxylase, the enzyme isoforms responsible for producing catechol estrogens. These inhibitors can control the formation of CE in target organs and will be helpful in further elucidating the biochemical roles of CE. We have optimized novel synthetic methods for synthesis of catechol estrogens which were reported in the annual report dated July 97.

Recently 2-Methoxyestradiol was reported to inhibit tubulin polymerization and act as an antiangiogenic agent. We evaluated a series of estrogen analogs as potential inhibitors of tubulin polymerization. In our preliminary screen we have identified 2-methoxymethyl estradiol as more potent inhibitor of tubulin polymerization than 2-methoxy estradiol. The SAR information derived from the above series along with several literature reports is being used to direct synthesis of non-steroidal agents as tubulin polymerization inhibitors. To this end we are developing novel synthetic routes that would enable us to synthesize combinatorial libraries of variously substituted benzopyrones. Synthesis and screening benzopyrone combinatorial libraries could result in identification of novel small molecule agents for biochemical targets important in controlling and treating breast cancer.

Abbreviations: E_1, estrone; E_2 estradiol; CE, catechol estrogen; HE_2, hydroxy estradiol: HME_2, hydroxymethyl estradiol; ME_2, methoxy estradiol; LP, lactoperoxidase; CT, calf thymus; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; QH_2, dihidroquinone; SQ, simiquinone; Q, quinone; ROS, reactive oxygen species; 2'-dG, 2'-deoxyguanosine; 8-oxo-dG, 8-oxo-deoxyguanosine; E½, half wave potential; Ep,a, anodic peak potential; Ep,c, cathodic peak potential; PUFA, polyunsaturated fatty acid, BCS, bathocuproinedisulfonic acid; GSH, glutathione, SOD, superoxide dismutase; ER, estrogen receptor

BODY

Part A

In Vitro Measurement of Oxidative DNA damage by Catechol Estrogen and Analogs

Introduction

The exact mechanism for estrogen's involvement in tumor development remains to be elucidated. Estrogens act as promoters via binding to nuclear estrogen receptors. There is increasing evidence that the catechol estrogens (CE's) may be involved in breast cancer initiation (1). Extensive metabolism of estradiol (E_2) and estrone (E_1) lead to CE formation in the liver and to a much lesser extent locally in breast tissue. The cytochrome P450 isoforms responsible for CE formation (ex: 1A1/1A2 and1B1) can be induced in MCF-7 cells by TCDD (2) and are often constitutively induced in human breast tumor tissues (3, 4). Cytotoxic levels of CE's transform cells in the BALB/c 3T3 assay (5) and are believed to be responsible for estradiol induced renal tumor formation in the Syrian hamster model (6). The CE genotoxicity mechanism may involve free radical generation by way of a $QH_2/^{\bullet}QH/Q$ redox cycling process (Figure 1). Compounds that contain an *o*- or *p*- dihydroquinone moiety, including the CE's, have been reported to redox cycle through processes driven by oxidoreductases, peroxidases, and metals such as copper (7, 8, 9). For example, lactoperoxidase, a breast tissue specific enzyme, is capable of oxidizing estradiol and is known to potentiate superoxide generation by *o*-hydroquinones (10, 11, 12). Copper has been found to potentiate CE mediated strand breaks *in vitro* (9), and EPR measurements have confirmed the presence of semiquinone and reactive oxygen species (ROS) in the cytosolic extracts from MCF-7 cells after CE exposure (13). There are numerous reports of CE induced DNA damage, which include DNA strand breaks

(14), 8-oxo-dG induction (6, 15, 16), and CE-DNA adduct formation (17, 18, 19). However, in all of the studies mentioned, non-physiologic levels of CE and in many cases a non-physiologic matrix (ex: organic solvents) were necessary to induce the DNA damage.

We previously reported the synthesis and biological evaluation of various 2- and 4-hydroxyalkyl estradiols as metabolically stable CE analogs. In order to gain further insight into the effects of CE induced DNA damage, comparison between hydroxy estradiols (HE_2's), methoxy estradiols (ME_2's) and hydroxymethyl estradiols (HME_2) were carried out in potentiometric and DNA damaging studies *in vitro*. In order to characterize the redox properties of these compounds the chemical reversiblities and half wave potentials were measured in a biological buffer at pH 7.4. The DNA damaging potential of the CE's was quantified with the genotoxic marker 8-oxo-dG in calf thymus DNA under the same conditions used in the potentiometric studies. 8-Oxo-dG induction has been correlated with CE exposure, is a DNA damage marker for oxidative stress (20), and is mutagenic, causing G-T transversion (21). This type of mutation has been found on the p53 gene in hepatic cells exposed to chronic inflammation (22). If handled properly, DNA can be digested without appreciable formation of background 8-oxo-dG or scission of the 8-oxo-dG ribosyl linkage. With the use of HPLC-ECD, 8-oxo-dG can be detected in the low femtomole range, making it a good marker for ROS generated DNA damage (23, 24). The process of DNA isolation, cellular DNA repair and the induction of antioxidant defenses make the measurement of 8-oxo-dG *in vivo* and in cell culture very difficult and often misleading (25). At present there is very little information regarding the formation of 8-oxo-dG by the CE's. For this reason we have examined the

mechanism of 8-oxo-dG formation by the CE's *in vitro* with the hope of transferring this knowledge to a cell culture system.

Results and Discussion

Measurements of Oxidation/ Reduction Potentials. Both 2-HE_2 (Figure 2a) and 4-HE_2 were quasi-reversible (Scheme 1a) with ΔE's of 55 mV and 60 mV respectively, and with nearly equal half wave potentials ($E_{1/2}$) of 263 ± 10 mV and 265 ± 10 mV vs the NHE, respectively (Table 1). 2-HME_2 (Figure 2b) and 4-HME_2 each exhibited one anodic peak at 597 ± 10 mV and 595 ± 10 mV vs the NHE respectively (Table 1) which was not reduced at the electrode surface. Although the end products were not studied, electrochemical investigations of polyalkylated phenols have been thoroughly reviewed (29). A reasonable mechanism under neutral conditions would include the loss of 2 e^- with generation of the phenoxonium intermediate followed by hydroxylation primarily in the one position. (Scheme 1b). This type of end product would lead to a CV profile consistent with that observed for the HME_2's. In order to show that the phenolic group was oxidized, a CV of 2,3-dihydro-pyranyl-estradiol was obtained with no oxidation peak within the water window (data not shown).

The methoxy metabolites were nearly identical (Figure 2c), each compound exhibited one anodic peak (Ep,a2) that decreased in current with each scan, and a second anodic peak (Ep,a1) followed by one cathodic peak (Ep,c1) that both increased in current with each scan. This CV was consistent with oxidation of the phenolic moiety followed by irreversible demethylation, and reversible reduction of the resultant quinone (Scheme 1c) (29). The Ep,a1 and Ep,c1 peaks were nearly identical to the oxidation and reduction peaks of 2-HE_2 (Table 1). The slight shift in $E_{1/2}$ may be due to generation of MeOH at the electrode surface. Therefore the very rate limiting process of spontaneous

demethylation would be necessary before any redox could occur. The half wave potential of the validation standard 4-methyl-catechol agrees within 30 mV of the literature value obtained under similar conditions (30).

8-oxo-dG Fomation by CE's and CE Analogs with and without Cu(II). Calf thymus DNA was exposed to 100 μM CE, CE analog and ascorbic acid, with and without 100 μM Cu(II)SO_4 for 3 hours (Figure 3). Ascorbic acid is known to increase 8-oxo-dG *in vitro* and was therefore used as a positive control (30). Cu(II)SO_4 was included in the experiments due to the noted redox coupling with hydroquinones, the low μM concentrations found in normal serum, and the reported association with histone proteins and dGuo in DNA (8, 23, 31, 32).

There was a slight increase in 8-oxo-dG formation over the non-incubated DNA (7.3 ± 0.9) for the various negative controls which included DNA incubated alone (11.7 ± 1.0), DNA + 100 μM Cu(II)SO_4 (14.3 ± 2.0), and the addition of DMSO which had no effect on the system. Increases in 8-oxo-dG over controls were significant for 2-HE_2 (21.0 ± 0.6), 4-HE_2 (15.5 ± 1.7) and ascorbic acid (31.3 ± 3.0). The addition of Cu(II) significantly increased 8-oxo-dG levels in the 2-HE_2 (1189 ± 119.3), 4-HE_2 (1256.4 ± 74.9) and ascorbic acid (1016.3 ± 89.0) samples over those samples containing no Cu(II). The HME_2's and ME_2's did not increase 8-oxo-dG levels even with the addition of Cu(II), and actually appear to behave in a protective fashion with less DNA damage than that found in the incubated controls. Although 2-HE_2 induced significantly more 8-oxo-dG than did 4-HE_2 when incubated alone, both CE's generated nearly equal DNA damage when Cu(II) was added. Cu(II) increased the DNA damaging potential of both CE's by

nearly 50 fold, illustrating the great significance of copper on CE toxicity. These results are in good agreement with what would be predicted from the potentiometric data.

Identification of ROS Formed by CE and Cu(II). Specific ROS inhibitors were chosen in order to help elucidate the damaging mechanisms that are especially significant in cells and *in vivo*. It is important to determine the type of ROS generated, as this can help predict other types of damage that may occur in proteins, PUFA's, carbohydrates and DNA (33, 34). Inhibition of ROS was measured as a percent decrease in 8-oxo-dG formed vs. a positive control containing 2-HE_2 and Cu(II)SO_4. Calf thymus DNA was exposed to 100 μM 2-HE_2 and 100 μM Cu(II)SO_4 for 3 hours (Figure 4) with the prior addition of specific ROS inhibitors as indicated. Concentrations are as follows: GSH (1.0 mM), NaN3 (1.0 mM), BCS (200 μM), MgCl2 (1.0 mM), SOD (200 U), Mannitol (1.0 mM), Catalase (200 U).

There was a similar decrease in 8-oxo-dG formation as compared to the positive control for the copper chelator bathocuproinedisulfonic acid (BCS) (166.0 ± 76.4, 86 % decrease), the copper chelator/ reducing agent glutathione (GSH) (458.7 ± 61.2, 61 % decrease), and in the anaerobic system (289 ± 15.6, 76 % decrease). GSH is mostly noted for its radical scavenging abilities, however GSH is also known to chelate metal ions such as copper (8). In the BCS copper ion complex, the formal potential is raised such that Cu(II)-BCS is easily reduced forming the Cu(I)-BCS complex which is difficult to oxidize rendering the copper ineffective in activating O_2. The high efficacy of the copper chelators along with the free radical scavenging activity of GSH point to a mechanistic role for the Cu(I)/Cu(II) couple as a 1 e- transfer agent initially causing formation of the CE semiquinone and superoxide radical anion. The significant decreased in 8-oxo-dG

formation in the anaerobic system reiterates the necessity for O_2 in this system. Sodium azide acting primarily as a $^1O_2$ scavenger (1247.3 ± 58.7, 5 % decrease), and mannitol as an $^{\bullet}OH$ scavenger (1173.3 ± 44.6, 1 % decrease) were far less effective at lowering 8-oxo-dG formation. However, catalase a H_2O_2 scavenger (26.7 ± 5.0, 98 % decrease) and SOD a $O_2^{\bullet-}$ scavenger (6.8 ± 0.8, 99 % decrease) lowered 8-oxo-dG to nearly that of background. The high efficacy of catalase and SOD indicate that peroxide and superoxide are the primary reactive oxygen species formed in this system. The less efficacious singlet oxygen and hydroxy radical scavengers indicate that these reactive oxygen species may be formed secondarily through Heiber-Wiese and Fenton chemistry at a very close proximity to the DNA. Magnesium and calcium (data not shown) were also added to the buffer separately and were found to have no affect on 8-oxo-dG production. Various biological buffers have been reported to decrease 8-oxo-dG formation in the H_2Q-Cu(II)-DNA system which is believed to be due to complexation with Cu(II) (12). We have found that magnesium and calcium often alleviate this effect possibly through competitive interactions with anionic sites in the buffer and possibly on the CT DNA which is usually purchased as sodium salt.

Concentration Requirements for Oxidative Induced DNA Damage. Numerous studies have shown that CE's are capable of inducing DNA damage at high concentrations. However, the minimal concentrations needed to induce such damage often goes unreported. In this study calf thymus DNA was exposed to increasing concentrations of 2-HE_2 (0.1 μM to 100 μM) with and without the addition of 10 μM Cu(II)SO_4 for 3 hours (Figure 5). The induction of 8-oxo-dG by 2-HE_2 was compared to non-incubated DNA, DNA incubated alone, or DNA incubated in the presence of Cu(II)SO_4. High

concentrations of 2-HE_2 induced 8-oxo-dG formation over incubated controls (carrier, 11.0 ± 0.2; carrier + Cu(II)SO_4, 13.4 ± 0.6) at no less than 100 μM alone (13.2 ± 0.6) and no less than 10 μM in the presence of Cu(II)SO_4 (45.7 ± 1.1). The addition of lower concentrations of 2-HE_2 decreased 8-oxo-dG formation to those levels found in the non-incubated control (7.0 ± 0.2) at 1.0 μM (7.6 ± 0.2) and 10 μM (7.6 ± 0.2) when added alone and at 0.1 μM (9.8 ± 0.4) and 1.0 μM (10.6 ± 0.1) with the addition of Cu(II)SO_4. These results are reinforced by reports that 2-HE_2 exhibits antioxidant activity in lipid peroxidation studies (35). Our data indicates that 2-HE_2 is primarily oxidized through a 2 e- transfer mechanism as may be expected from the CV experiments measured under aqueous conditions at pH 7.4. Although reduction of the resultant peroxide molecules would be expected to proceed similarly, the addition of Fenton catalysts such as copper and the reaction of residual superoxide through a Haber-Weiss mechanism would produce DNA damaging hydroxyl radicals. This would explain the antioxidant effects that are seen at lower concentrations of 2-HE_2 and why high concentrations of CE and the addition of copper are necessary before any oxidative DNA damage occurs.

Lactoperoxidase Effects on the DNA Damaging Potential of CE's. The LP catalyzed $1e^-$ oxidation of estradiol and *o*-quinones have been reported, this has inspired us to look at 8-oxo-dG formation by CE in the presence of LP (11, 12). Calf thymus DNA was exposed to 100 μM of either 2-HE_2 or 4-HE_2, 2 units of lactoperoxidase (LP) and 100 μM H_2O_2 for 3 h.

Controls were performed with the addition of either H_2O_2 alone or H_2O_2 with 2-HE_2. There was a significant increase in 8-oxo-dG formation by the 2-HE_2 and peroxide control (50.0 ± 2.0), with levels decreasing to that of background for both CE's (6.5 ±

0.4, 2-HE_2 and 6.2 ± 0.9, 4-HE_2) when LP was added. The results in Table 2 indicate that LP may behave in a protective fashion. The 1 e^- oxidation pathway by LP would produce the CE semiquinone and resultant ROS with expected increases in 8-oxo-dG, which is not observed. This leads us to believe that the peroxide driven CE oxidation by LP proceeds through a 2 e^- transfer mechanism and not a 1 e^- transfer mechanism as may be expected. This is in partial agreement with spectroscopic studies, which illustrate accelerated oxidation of 2-HE_2 by LP (Figure 6). The spectroscopic studies also indicate that 2-ME_2 and 2-HME_2 are virtually inert toward the effects of LP (data not shown). Although LP has been shown to decrease CE induced oxidative DNA damage *in vitro*, the potentially toxic effect of the resultant electrophilic quinone should not be ignored.

Experimental Procedures

Materials and Methods. 8-Oxo-2'-deoxyguanosine (8-oxo-dG) was prepared as described below. The 2- and 4-HE_2's and ME_2's were purchased from Steraloids. The 2- and 4- HME_2's were prepared as previously described (26, 27). Calf thymus DNA, 4-methyl-catechol, 2'-deoxyguanosine, NH_4Ac, ascorbic acid, GSH, mannitol, H_2O_2, alkaline phosphatase, and lactoperoxidase were all purchased from Sigma Ltd. Nuclease P1 was purchased from Boeringer Mannheim.

8-Oxo-2'-Deoxyguanosine Synthesis and Purification. 8-Oxo-dG was prepared according to the method of Kasai and Nishimura (28) with modifications. To 100 ml of a 0.4 mM solution of 2-dG dissolved in a 0.5 M $NaPO_4$ buffer at pH 6.5-7.0 was added 10 eq. of a 3 % solution of H_2O_2 and 0.5 eq. ascorbic acid. The reaction mixture was stirred vigorously at 37°C as a steady stream of O_2 was bubbled into the mixure for the duration of the reaction. After 2 hours, the same portions of H_2O_2 and ascorbic acid were added for a second time as above. The reaction was stirred for 2 more hours and then concentrated to 15 ml at 40°C (higher temps can cause degradation and complete evaporation can form insoluble phosphate complexes). Purification was carried out with a preparative C-18 column, 4.0 ml/min., 3 % MeOH in H_2O, UV detection at 254 nm (8-oxo-2-dG elutes directly after 2-dGuo). Yields varied from 8-12 % after purification. Identification was confirmed by ^{1}H-NMR (DMSO-d_6, 250MHz) and coelution with a standard by HPLC.

Potentiometric Measurements. Potentiometric measurements were performed on a BAS CV-1B cyclic-voltammeter. The sample cell included the platinum auxiliary, glassy

carbon working and Ag^+/AgCl reference electrodes. The sweep rate was optimized at 100 mV/s, with a sensitivity of 2 μA/V and a filter rate of 0.1 s. Sample solutions were made with the addition of 10 mM stocks in DMSO into an N_2 purged cell containing PBS (pH 7.4, Ca/ Mg free) to give 100 μM working solutions. It was necessary to polish the glassy carbon electrode after each scan and 4-methyl catechol served as a standard for system validation.

***In Vitro* DNA Damage Experiments.** Freshly prepared 1 μg/ μL solutions of calf thymus DNA (200 μl) in PBS (Ca^{2+}/ Mg^{2+} free at pH 7.4) were delivered to uncapped 12x75 mm borosilicate glass culture tubes. The compounds of interest were added and incubation was carried out in a shaking water bath at 37°C for 3 hours. The steroid stock solutions were made up in DMSO, while all other compounds were diluted in DI water. Stocks were added to the CT DNA solutions such that a 1:100 dilution of the stock solution would give the appropriate working concentration. DNA was precipitated with the addition of 50 μL 7 M NH_4Ac and 500 μL of chilled 95 % EtOH, and treated as indicated in the DNA digestion procedure.

Enzymatic DNA Digestion. The precipitated DNA was transferred to a 0.5 ml heat resistant spin tube and pelleted. The resultant pellet was washed with 70 % EtOH followed by 90 % EtOH, allowed to air dry, inverted for 10 minutes and diluted with 200 uL of a 10 mM TRIS solution at pH 7.0. The DNA was denatured at 95°C for 10 minutes and cooled on ice. This was followed by the addition of 10 units of nuclease P1 in 20 mM NaAc buffer at pH 3.4 (resultant pH 4.8) containing 0.1 mM $ZnCl_2$, and heating to 65°C for 15 min (incubation times longer that than 15 minutes can cause an increase in 8-oxo-dG). The solution was cooled to 37°C and 20 units of alkaline phosphatase type VII-S

was added in 200 mM Tris at pH 8.5 (resultant pH of 7.8) and digested for 45 minutes. The final solution was pH adjusted to 6.0 with 0.05 M HCL and injected on the HPLC/ECD system.

HPLC/ECD System. Separation of the hydrolyzed DNA was achieved on a Beckman HPLC model 126 containing an ESA pulse dampener just prior to the sample injector and a YMC basic 5 μm, B-02-3, 15 cm reverse phase column. The optimum isocratic system contained 5% MeOH in 0.1 M $NaPO_4$/ 0.1 M NH_4Ac buffer at pH 6.0 with a flow rate of 0.8 ml/minute. Detection was carried out using a Beckman UV detector model 167 at 260 nm and an ESA Coulochem Detector II with guard cell removed, conditioning cell at 100 mV and sample cell at 350 mV. Data acquisition was carried out on a Metrabit analog to digital converter.

Appendix 1

Tables and Figures

Table 1. Peak Potentials for CE's and CE Analogs vs. Ag/AgCl at pH 7.4

	Peak potentials are expressed in mV					
compound	Ep, a1	Ep, c1	Ep, a2	ΔE	E½[a]	E½[b]
2-OH-E_2	90	35	-	55	73	263
2-MeO-E_2	80	15	280	65	48	238
2-HMe-E_2	407	-	-	-	-	-
4-OH-E_2	95	35	-	60	75	265
4-MeO-E_2	80	25	345	55	53	243
4-HMe-E_2	405	-	-	-	-	-
4-Me-Catechol	140	50	-	90	95	285

[a] Std. Err. ± 10 mV, [b] Potentials corrected vs. NHE at pH 7.4

Table 2. Lactoperoxidase Effects on CE Induced 8-oxo-dG Formation

Experiment	8-oxo-dG/10^5 dG
CT DNA only	7.3 ± 0.9
H_2O_2 100 μM	17.0 ± 0.3
H_2O_2 100 μM + 2-HE_2 100 μM	50.0 ± 2.0
LP 2 U + H_2O_2 100 μM + 2-HE_2 100 μM	6.5 ± 0.4
LP 2 U + H_2O_2 100 μM + 4-HE_2 100 μM	6.2 ± 0.9

Data points are means of N=3 ± SD for all samples

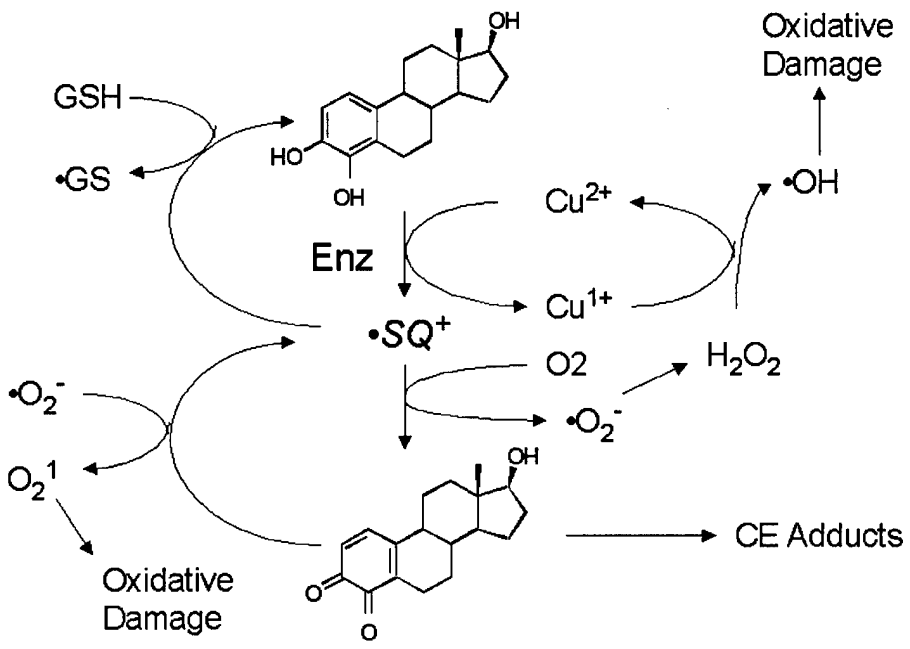


Figure 1. Proposed Genotoxicity Mechanism for Catechol Estrogens

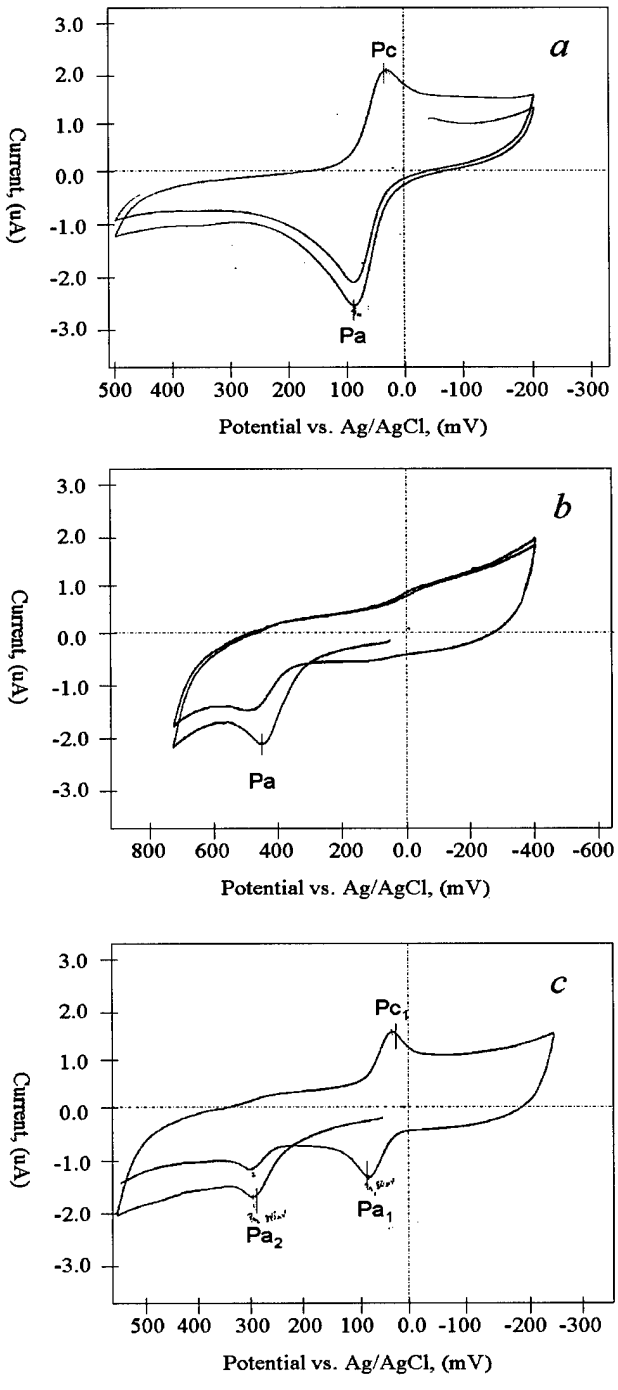


Figure 2. CV of (a) 2-HE_2, (b) 2-HME_2 and (c) 2-ME_2

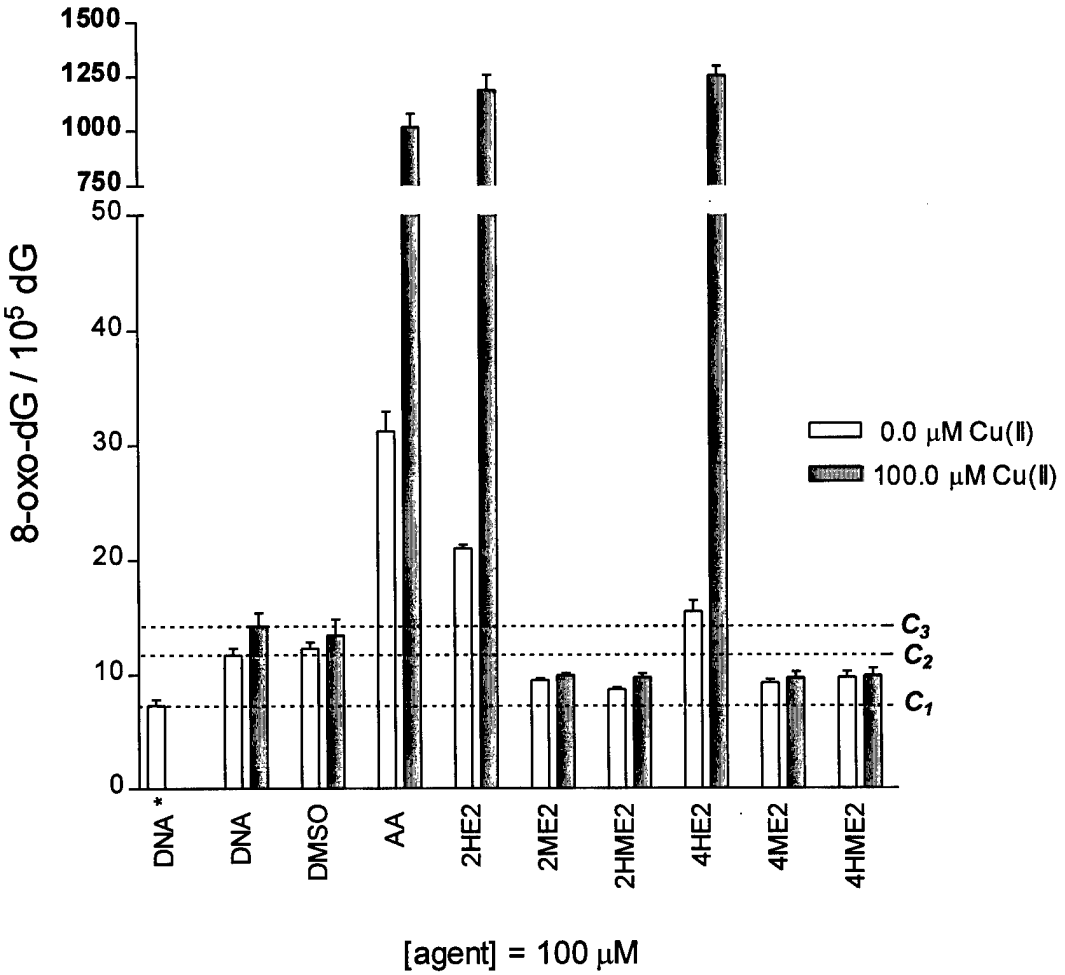


Figure 3. Calf thymus DNA was exposed to 100 μM of CE or CE analog with and without the addition of 100 μM Cu(II)SO_4 for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG was compared to that of the following controls; non-incubated DNA* (C_1), DNA incubated alone (C_2), and DNA incubated in the presence of Cu(II)SO_4 (C_3). Data points are means of N=3 ± SD for all samples.

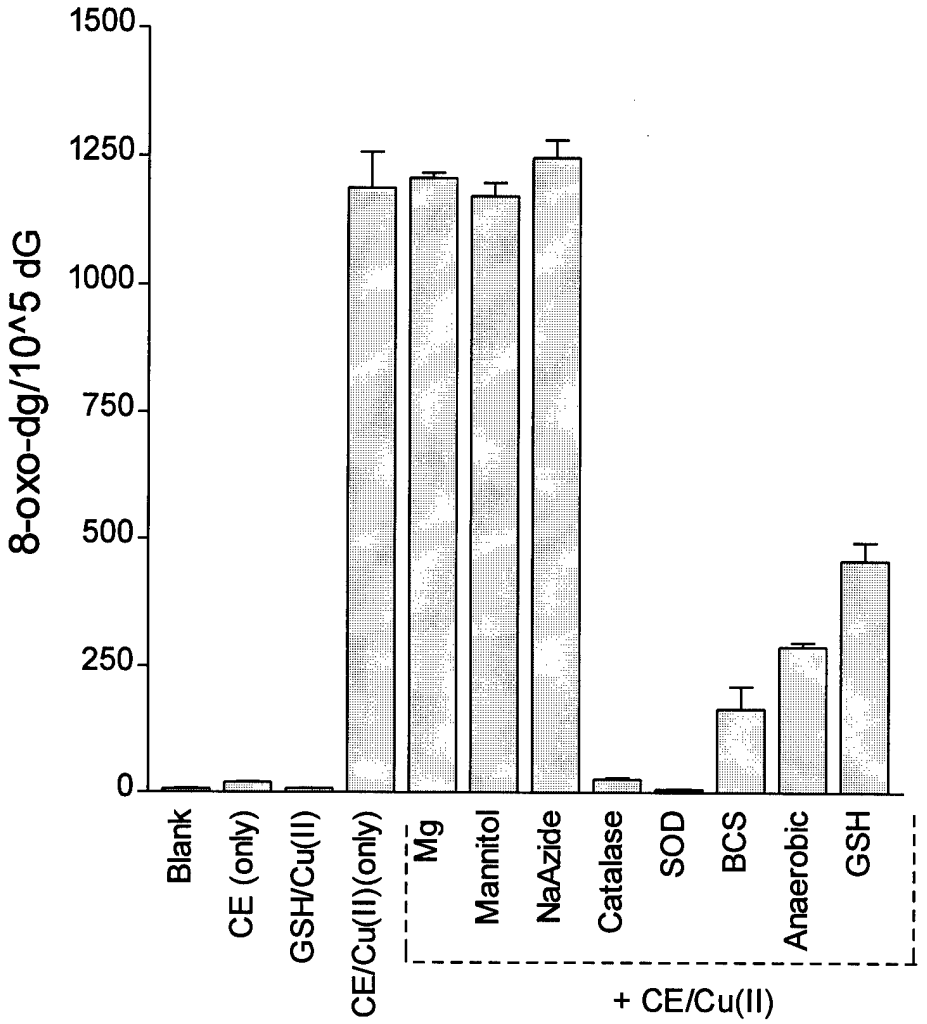


Figure 4. Determination of ROS Production by 2-HE_2 (100 μM) in the Presence of Cu(II) (100 μM). Samples were incubated for 3 h in PBS (pH 7.4) at 37°C in the presence of a copper chelator or ROS quenching agent as shown. Concentrations are as follows: GSH (1.0 mM), NaN3 (1.0 mM), BCS (200 μM), MgCl2 (1.0 mM), SOD (200 U), Mannitol (1.0 mM), Catalase (200 U). Data points are means of N=3 ± SD for all samples

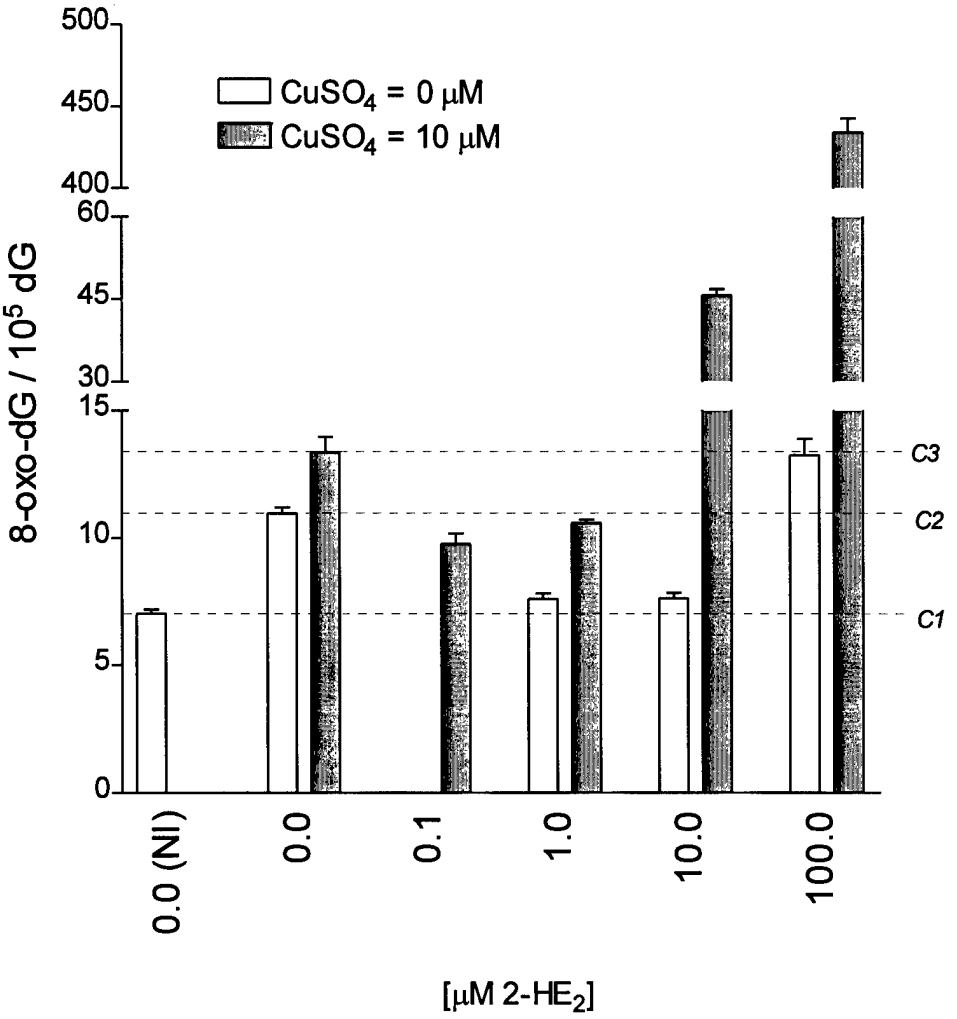


Figure 5. Calf thymus DNA was exposed to increasing concentrations of 2-HE_2 (0.1 μM to 100 μM) with and without the addition of 10 μM Cu(II)SO_4 for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG by 2-HE_2 was compared to that of the following controls; non-incubated DNA* (C_1), DNA incubated alone (C_2), and DNA incubated in the presence of Cu(II)SO_4 (C_3). Data points are means of N=3 ± SD for all samples.

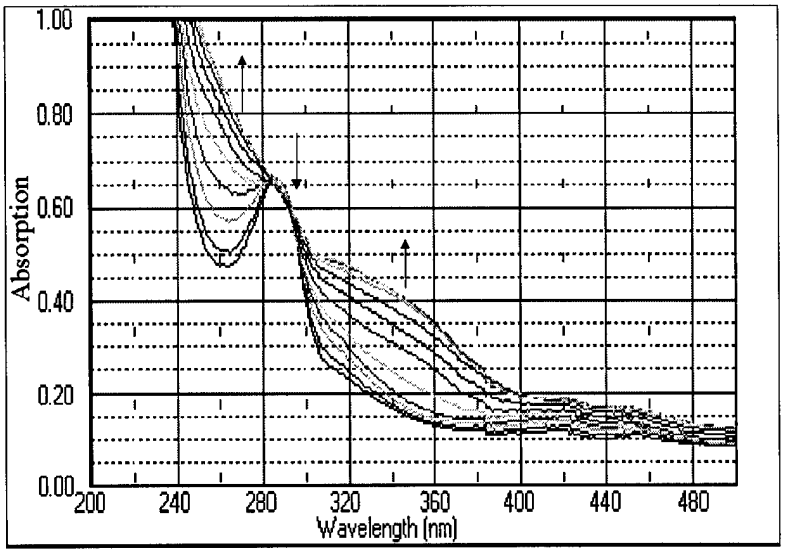
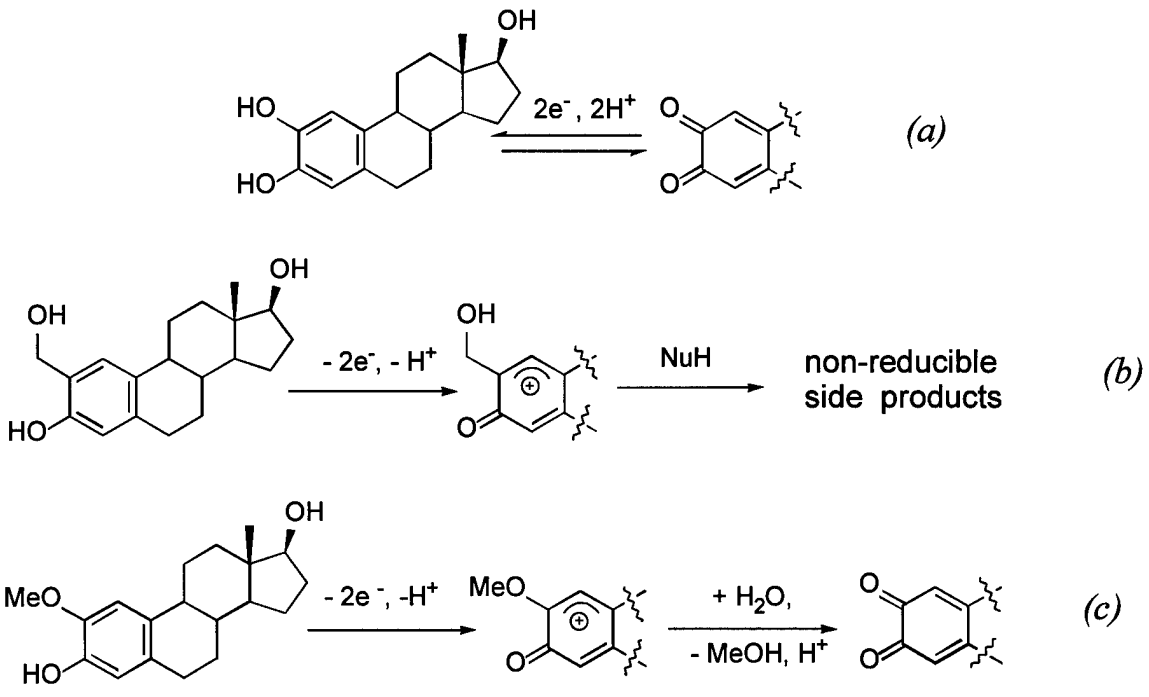


Figure 6. Overlapping absorption spectra of 2-HE_2 (100 μM) as it become increasingly oxidized by LP (2 U) and H_2O_2. H_2O_2 (10 μM) is added every 2 minutes while scans were taken every 4 minutes.



Scheme 1. *(a)* Reversible oxidation/ reduction of 2-HE_2, *(b)* irreversible oxidation of 2-HME_2, *(c)* oxidative demethylation of 2-ME_2.

Part B
Chemistry

Introduction

Chemical efforts in this project have concentrated on synthesis of stable catechol estrogen mimics. Synthetic approaches for making 4-hydroxyalkyl and 4-aminoalkyl estrogens were reported in last year's annual report. The work was subsequently published as an article in J.Med.Chem (1). A reprint of that manuscript is enclosed with this report.

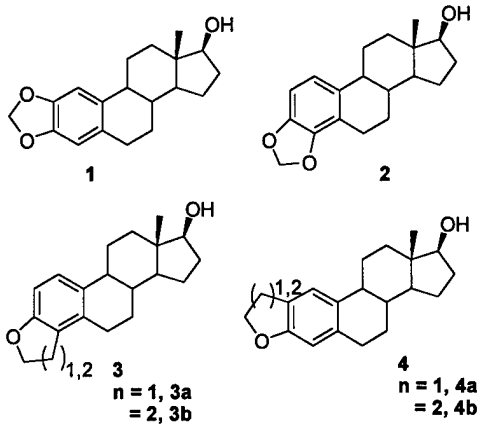
Synthetic work over the last year has concentrated on:

1) Synthesis of Estrogen A-ring fused analogs: 2,3- and 3,4-Methylenedioxy, 2,3- and 3,4- dihydropyranyl and dihydrofuranyl estradiols have been synthesized as potentially selective inhibitors of estrogen 2- and 4- hydroxylase, the enzyme isoforms responsible for forming Catechol estrogens.
2) Synthesis of Catechol Estrogens: Catechol estrogens are chemically unstable and easily decompose on air oxidation. The chemical instability makes catechol estrogens difficult to synthesize. Low yields and complex product mixtures mark the literature methods. We employ a Baeyer Villiger oxidation of formylestradiols to give protected catechols. The CE's are then produced under a reducing environment thereby circumventing oxidative complications.
3) Synthesis of Alkoxyalkyl estrogens: We have synthesized a series of 2-substituted alkoxyalkyl estradiols. These were evaluated as inhibitors of tubulin polymerization.
4) Novel synthetic approaches for benzopyrone nucleus: We are investigating various synthetic approaches for synthesis of benzopyrone combinatorial libraries. These libraries are to be screened for selective modulators of ERα and ERβ; inhibitors of

cellular tyrosine kinases, novel antitubulin agents, and antiflammatory agents. Thus we hope to identify novel thearpeutic agents for controlling and treating breast cancer.

Synthesis of Estrogen A-ring fused heterocycles:

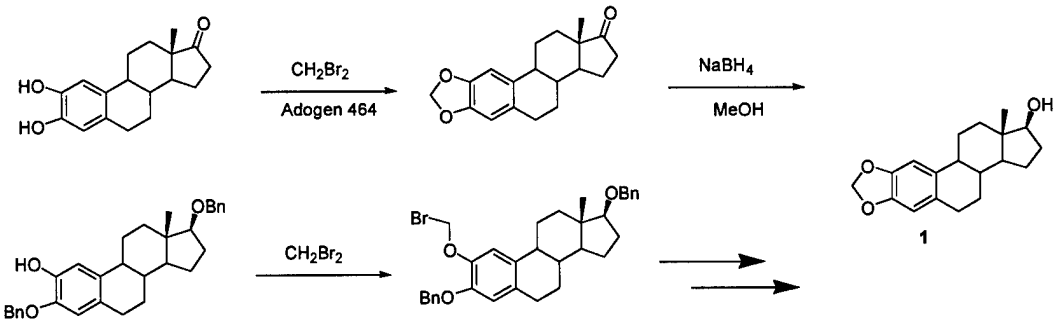
Estogens are hydroxylated at 2-and 4- positions by specific NADPH-dependent cytochrome P450 enzymes (2). Several extrahepatic tissues have been shown to express estrogen-hydroxylating enzymes. In the liver the catechols are readily methylated to form methoxy estrogens. However in the peripheral tissues the methylating enzymes are not readily available resultantly the catechol estrogens can stay around longer and exert biological effects (3). In order to understand this process it would be important to have compounds that could regulate the estrogen hydroxylating enzymes. Additionally if catechol estrogens are definitively implicated as tumor initiators, then compounds that inhibit the formation of catechol estrogens can prove important in controlling breast cancer. With these objectives we decided to synthesize and evaluate estrogen A-ring fused heterocycles as potential inhibitors of estrogen hydroxylase enzyme. Towards this end we have synthesized compounds shown below. These compounds have A-ring



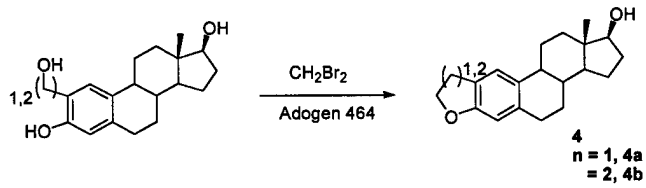
phenolic oxygens to facilitate enzyme recognition. These compounds will be evaluated as inhibitors of estrogen hydroxylase enzymes in microsomal preparations. The

methylenedioxy compounds were synthesized by treating the corresponding catechols with dibromomethane and Adogen 464, a phase transfer catalyst. Another method we are investigating involves alkylation of 2-hydroxybisbenzyl estradiol (see synthesis of catechol estrogen) with dibromomethane followed by deprotection and cyclization to give methylenedioxy analogs. This method eliminates the need to make and handle the highly labile catechol estrogens.

Synthesis of Methylenedioxy analogs



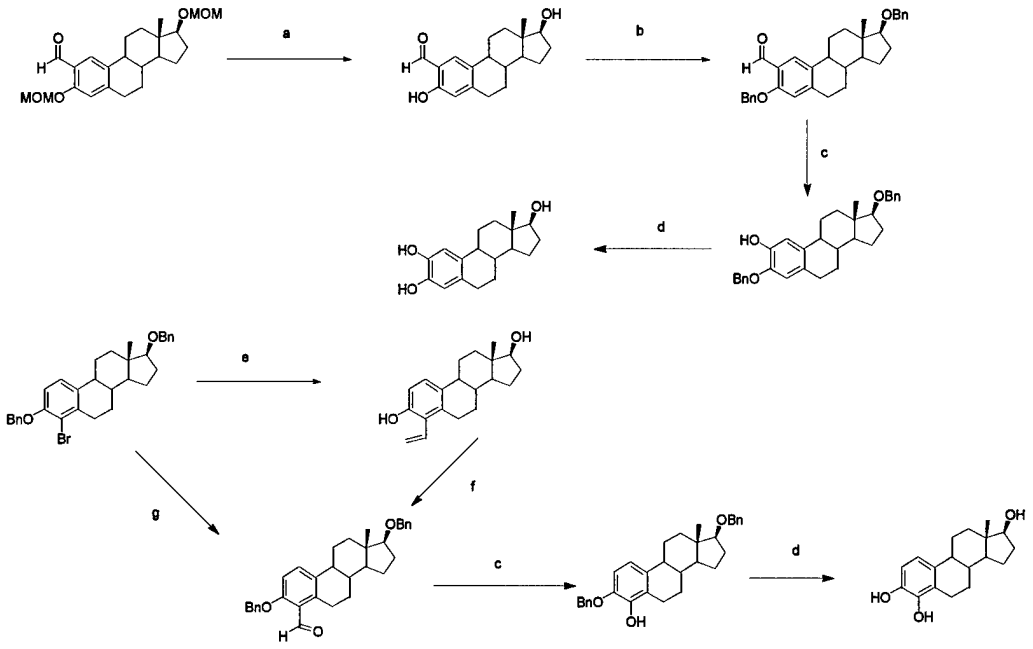
Synthesis of Dihydro pyranyl and Dihydrofuranyl analogs



Hydroxyalkyl estradiols obtained by methods reported in our previous publications were subjected to cyclodehydration reaction with PPh_3 and DEAD resulted gave the dihydropyranyl, dihydrofuranyl analogs in excellent yields.

Synthesis of Catechol Estrogens

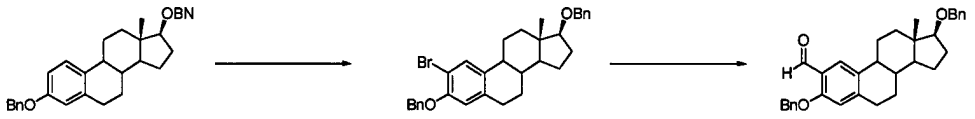
Catechol estrogens are chemically unstable and easily decompose by air oxidation. The chemical instability makes catechol estrogens difficult to synthesize. In the Annual report dated July 1997, we had reported on novel synthesis schemes for synthesis of catechol estrogens. Over the last year the synthesis has been optimized and is shown in the scheme below:



Reagents and Conditions: (a) 6M HCl, THF, 98%; (b) NaH, TBAI, BnBr, DMF, 65%; (c) MCPBA, pTSOH, CH_2Cl_2, 73%; (d) 10% Pd-C/H_2, 80%; (e) $Pd(PPh_3)_4$, Vinyltributyltin, DMF, Δ,85%; (f) (i) O_3, -78^{0}C, MeOH-CH_2Cl_2; (ii) DMS 78% ; (g) (i) n-BuLi, -78^{0}C, 2hr; (ii) freshly distd. DMF, -78^{0}C→0^{0}C, H^+, 37%

By using the above shown schemes we were able to generated 2- and 4- hydroxy estradiols in good yield and sufficient purity. However, it was noted during the course of the synthesis that the intermediates required for synthesis of 4-hydroxyestradiol are extremely unstable and require very careful handling. As mentioned in previous report

we attempted Pd-catalyzed CO insertion reactions to synthesize the formyl derivatives in a single step from the corresponding halo estrogens. However, these efforts were not successful. Recently, Cushman and coworkers have shown that by treating bisbenzyl-protected estradiol with bromine in acetic acid it is possible to isolate 2-bromobisbenzyl protected estradiol (4). The 2-bromobisbenzyl estradiol is subjected to halogen lithium exchange by treating with n-BuLi and subsequent trapping of the aryl-lithium with freshly distilled DMF yielded the 2-formylbisbenzyl estradiol in excellent yields. We now use this method to generate 2-formylderivatives as precursors for the 2-hydroxyestradiol synthesis.



We attempted several methods to optimize the synthesis of 4-formyl estradiol. As mentioned earlier the CO insertion reactions failed. We can use the 4-bromobisbenzyl protected estradiol in a Stille coupling with vinyltributylstannane to give 4-vinyl bisbenzyl estradiol in >85% yield. This when subjected to ozonolysis and reductive workup with methylsulfide gives the 4-formylestradiol in 75% yield in two steps from the bromo compound. In order to access the formyl compound in a single step from the 4-bromo derivative we attempted the strategy used for the 2- formyl compound, namely halogen-lithium exchange and quenching with freshly distilled DMF. In case of the 2-bromo estradiol this reaction worked extremely well yielding the formyl compound in 80% yield. However, the 4-formyl estradiols could be obtained in only 40% yield. It is interesting to note that the 4-formyl estradiol is very unstable and undergoes decomposition upon standing. We found it necessary to carry the 4-formyl estradiol into

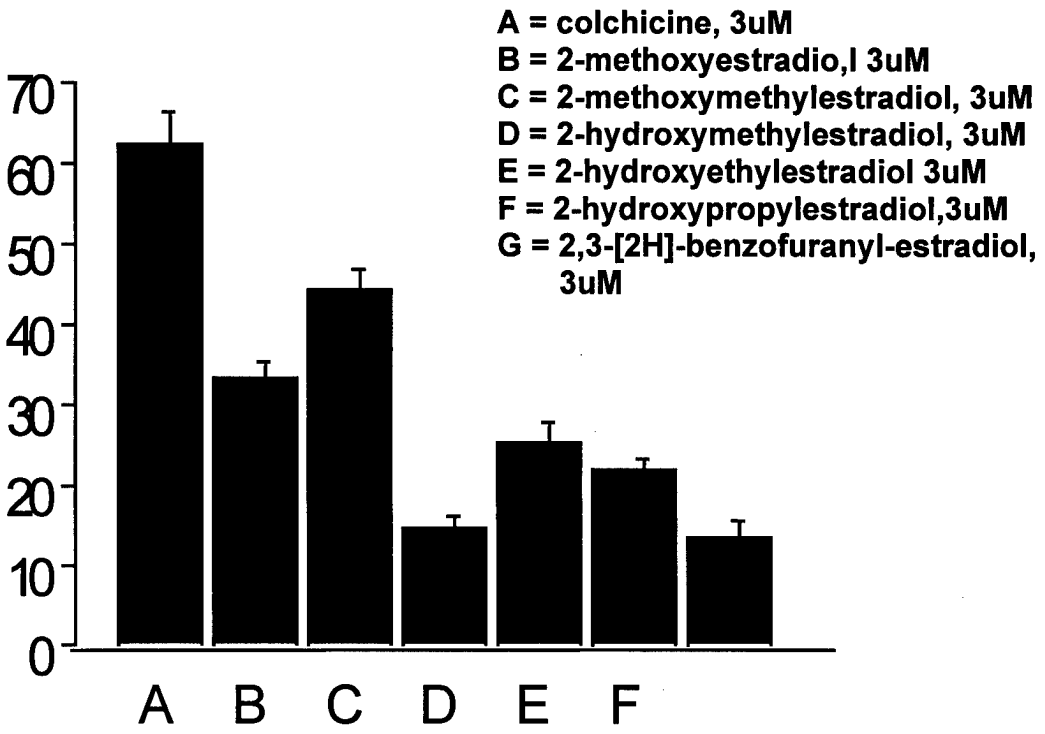
the next step as soon as it was prepared. The synthesis of 2-hydroxy estradiol went very smoothly, however the 4-hydroxyestradiol series caused considerable problems because of instability of the intermediates. It would be interesting to explore the chemical reasons behind the stability differences of the 2- Vs 4- series of compounds reported in the above scheme. This could shed some light on the reports that 4-hydroxy estradiol being a carcinogen while 2-hydroxyestradiol is considered benign.

Synthesis of alkoxyalkyl estrogens:

Functional microtubular system is required to maintain normal cell growth and function. Microtubules are involved in intracellular transport phenomena, required for mitotic spindle formation, cell division and they also function as cytoskeletal elements . Microtubules are formed by polymerization/aggregation of heterodimeric protein, tubulin. A number of natural products are known to interact with tubulin and disrupt the normal tubulin polymerization/ depolymerization events (5). Disruption of microtubules arrests cell growth and proliferation and therefore is an attractive biochemical target for development of antineoplastic agents.

D'Amato *et al.* have shown that 2-methoxyestradiol inhibits *in vitro* tubulin polymerization by interacting at the colchicine binding site (6). Sato *et al.* on studying effects of 30 natural steroids in a Chinese hamster V79 cell line, identified 2-methoxy estradiol as the most potent microtubule disruptive agent amongst the steroids studied (7). Interestingly, Fotsis *et al.* have reported that 2-methoxy estradiol inhibits angiogenesis and suppresses tumor growth (8). They suggest that abnormal microtubule assembly may be responsible for the angiogenic activity. Cushman *et al.* have looked at a series of 2-alkoxy, 2-alkyl and 2-thioalkyl derivatives and identified 2-ethoxyestradiol and 2-propylestradiol to be more potent tubulin polymerization inhibitors than 2-methoxyestradiol (9).

The steroidal nucleus seems to be an attractive target for developing novel tubulin polymerization inhibitors. Additionally, such steroidal compounds may have low- toxicity and as compared to the natural products known to interact with tubulin. We decided to evaluate the 2- and 4-hydroxyalkyl derivatives and their corresponding methyl ethers as potential tubulin polymerization inhibitors. Refer to last years Annual Report for details on synthesis of these compounds. The results from our preliminary screening efforts are shown below:

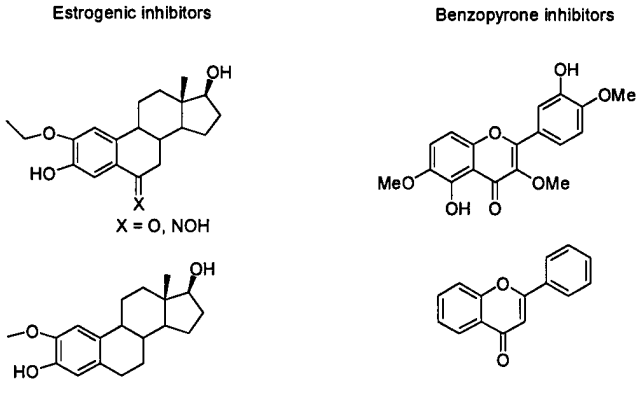


Method (10):

Tubulin solution (240 µl in MES buffer, pH 6.5, 0.5mM Mg^{+2}, conc. 1mg/ml) was incubated with 10µl of drug solution in DMSO at 37^{0}Cfor 15mins. The samples were chilled on ice and GTP was added (2.5 µl of 100mM solution) . Reaction mixtures were transferred into cuvettes chilled on ice, base line was established and polymerization was followed @ 350nm for 45mins with data points being sampled every 90secs. % Inhibition of assembly after 20 min of incubation was used to compare potency of different drug solutions. All assays were run in tiplicates unless indicated.

Discussion:

We found that 2-methoxymethyl estradiol is a more potent inhibitor of tubulin polymerization than 2-methoxy estradiol. Recently, Cushman *et.al.* have shown that 2-ethoxy, 6-oxo or 6-oximino estradiols are potent inhibitors of tubulin polymerization (4). This report came at about the same time that we were looking into the possibility of transferring the SAR from the estradiol series and making nonsteroidal antitubulin agents. Several molecules with benzopyrone ring system have shown to be ligands for the estrogen receptor (11a). Additionally, there are literature reports of some benzopyrones being active as tubulin polymerization inhibitors (11b). Interestingly if we compare the



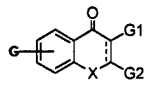
benzopyrones with Cushman's 6-oxo estradiols, we can readily see that the 6-oxo group in these inhibitors corresponds with the 3-keto group of the benzopyrones. The benzopyrone ring system provides an ideal template for nonsteroidal inhibitors of tubulin polymerization. We reasoned that the benzopyrone ring system could be exploited to develop novel, potent antitubulin agents. Additional literature survey indicated that the benzopyrone ring system is present in a number of natural products including flavonoids. They form a biologically interesting group of molecules interacting with a number of

enzymes and receptors of pharmacological significance (12). Some of these compounds have shown activity as tyrosine kinase inhibitors, PKC inhibitors, Antiinflammatory, Antiangiogenic agents, and Antiestrogenic agents (13). These represent important molecular targets for developing new therapies for controlling and treating breast cancer. So we decided to exploit the benzopyrone nucleus on a broader perspective using the modern combinatorial chemistry techniques. The details of the development of this project are given in the following section.

Novel Synthetic Approaches for Benzopyrone Combinatorial Libraries:

The field of drug discovery and design has witnessed a conceptual revolution with the advent of combinatorial chemistry technology (14). The past couple of years have witnessed a growing effort to develop new synthetic routes and analytical techniques to construct combinatorial libraries of small heterocyclic molecules (14,15). There is particular interest in using combinatorial chemistry to construct libraries of the so-called "Privileged Structures" (16). These libraries promise to provide ligands for a diverse collection of biological targets.

Benzopyrone ring system is a "Privileged Structure" that as yet remains to be exploited by combinatorial chemistry. It represents a fairly rigid molecular framework, resistant to hydrophobic collapse and with multiple sites of potential diversity. Benzopyrone ring system is present in a number of natural products, forming a biologically interesting group of molecules that interact with various enzymes and receptors of pharmacological significance (12,13). Thus, the low molecular weight, fairly rigid benzopyrone nucleus should serve as an ideal template for combinatorial libraries. We believe that the **synthesis and screening of substituted Benzopyrone combinatorial libraries would allow us to harvest the biological potential of these molecules.**

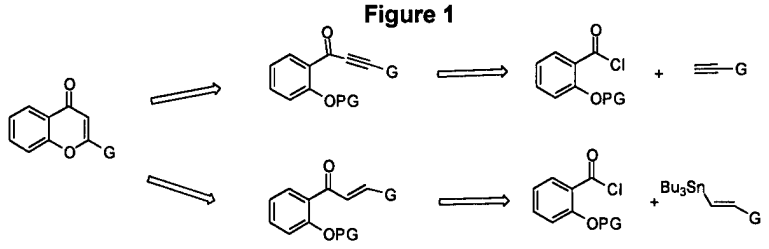


X= O, N, S
G= Alkyl, OH, OR, NHR,etc.
G1,G2 = aromatic, heteroaromatic, alkyl, cycloalkyl.

Design of Synthetic Routes for Constructing Libraries

Prevalent literature methods were not ideally suited for making benzopyrone combinatorial libraries. The existing protocols suffer from harsh reaction conditions, low yields and poor

Figure 1



substituent tolerance. Additionally, the literature methods represent two component condensation reactions with limited use in making diverse libraries. Recently it was shown that heteroannulation reactions of terminal alkynes with *o*-iodophenols in presence of CO give mixtures of benzopyrones and aurones (17).

In our scheme by using salicyloyl chlorides instead of iodophenols we eliminated the high CO pressure conditions required for heteroannulation reactions. The benzopyrone ring was constructed by a 6-endo-dig cyclization of the alkynone under controlled reaction conditions that preclude the formation of aurones by the competing 5-exo-dig pathway. Generation of salicyloyl chlorides and coupling of alkynes to give alkynones followed by cyclization of the alkynones to give benzopyrones form the key reactions of the proposed scheme shown in Figure 1.

Synthesis of Salicyloyl Chlorides

The reaction scheme relies on readily available substituted salicylic acids and terminal alkynes as input building blocks. Salicylic acids are reacted with two equivalents of TBSCl to give bisTBS protected salicylic acids. These are converted into 2-OTBS Salicyloyl chlorides under neutral conditions using Wissner's method (18).

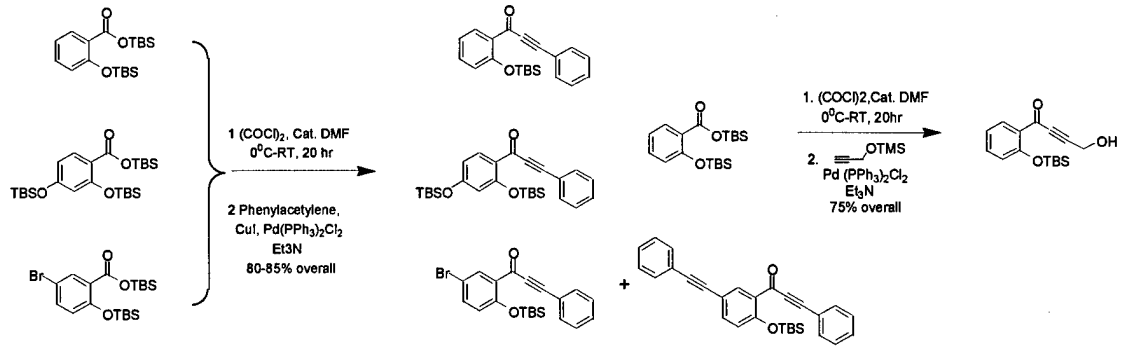
Heck and Stille couplings of the Acid Chlorides

The acid chlorides are directly carried over to the coupling reactions without any purification. For the coupling of terminal alkynes, Hagihara's method employing $Pd(PPh_3)_2Cl_2$ and CuI in an amine solvent worked the best in terms of substituent tolerance and overall yields (19). This is a key reaction for introducing diversity. Present work in the lab is focusing on understanding the stereoelectronic factors governing this reaction. These studies should define the nature of substituents that could be introduced for making diverse libraries. Alternatively, the acid

chlorides can be coupled with alkynyl/ alkenyl stannanes in presence of $Pd(PPh_3)_4$, CuI in toluene (20).

Figure 2

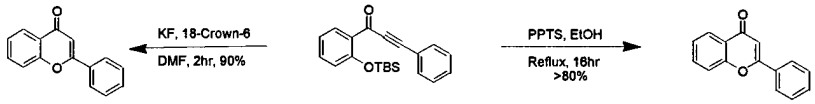
Coupling of Acid chlorides



Cyclization to Flavones

The alkynones were cyclized under basic conditions using KF and 18-crown-6 in DMF (21). The cyclization proceeds by a 6-endo-dig mode to give the flavones. The competing 5-exo-dig

Figure 3

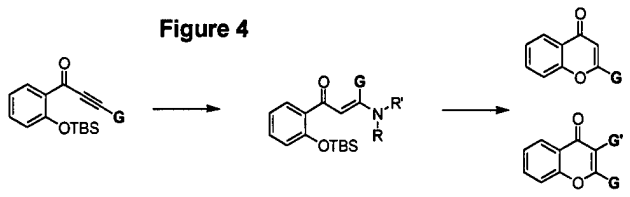


cyclization resulting in aurones was the predominant pathway only in presence of protic solvents. However, when the alkynone was refluxed with PPTS in MeOH to remove the TBS group, the free phenol cyclized under the reaction conditions to yield the flavone. No 5-membered aurones were detected under these conditions. Additionally, refluxing the alkynone with excess of dimethylamine for 10 hours produces the flavones in >70% yield.

Benzopyrones with substituents at 2- and 3- positions

Alkynones treated with secondary amines give the corresponding enaminoketones in excellent yields. These enaminoketones were cyclized under basic conditions to give flavones.

Figure 4

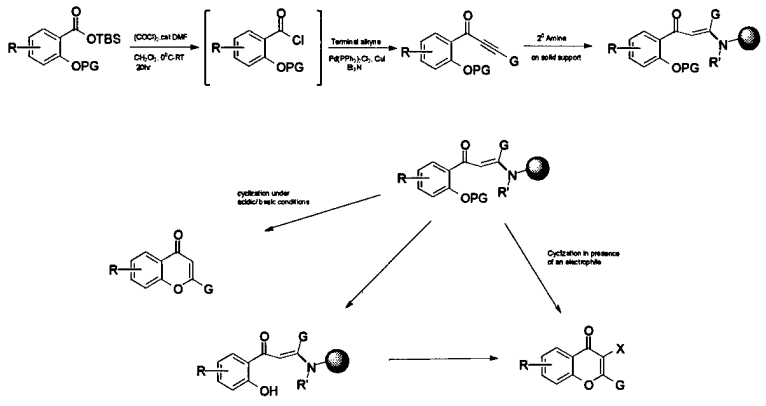


Additionally, we are investigating reaction conditions wherein the enaminoketones are cyclized in presence of a halogen source to yield 3-halo benzopyrones (22). 3-halobenzopyrones can be further functionalized using Suzuki coupling to yield ring systems with substituents at both the 2- and 3- positions (23). The input salicylic acid, terminal alkyne and the 3-halo position form the three points for introducing diversity.

Proposed plans for synthesis of Combinatorial libraries

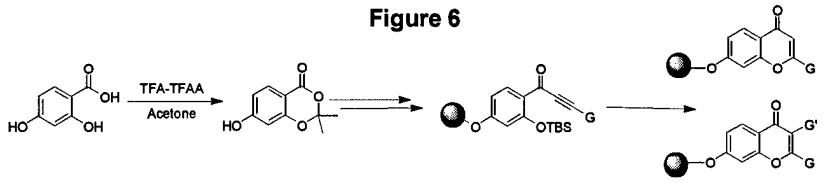
Several strategies for constructing libraries using chemical schemes discussed above are being investigated. Reactions of alkynones with secondary amines (Fig. 4) open up an opportunity to employ a resin capture strategy as shown in Figure 5. Thus, alkynones will be fished out of

Figure 5



reaction mixtures using support bound secondary amines. Reactive functionalities on these enaminones can be derivatized when bound to solid support, thereby increasing the library diversity. Subsequent cyclizations of support bound enamino ketones should exclusively release the 2 - or 2,3- disubstituted benzopyrones into the solution.

Figure 6



It is also possible to transfer this chemistry to solid phase as shown in Figure 6. Briefly, salicylic acid is protected as its benzacetonide in moderate yields (40-60%) (24). The benzacetonide will be linked to solid support and converted to the alkynone in 3 steps followed by subsequent cyclization. This reaction scheme has been carried out in solution phase successfully, and we want to study the feasibility of this reaction scheme on solid phase using a model system.

Conclusions

- Developed novel synthetic routes suitable for constructing benzopyrone libraries. Versatile reaction intermediates can be used for library synthesis of other ring systems.
- Presently investigating solution, resin capture and solid phase methods for library synthesis.
- The libraries are to be screened for selective modulators of ERα, ERβ and inhibitors of tubulin polymerization.

Chemistry Experimental details:

Estradiol was purchased from Steraloids (Wilton, NH). All other chemicals were purchased from Aldrich Chemical Co.(Milwaukee) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. Amines were stirred over CaH2, distilled and then stored over KOH pellets. Silica gel TLC plates (60 F254) were purchased from Analtech Inc. (Newark, NE) and visualized with a UV lamp and/or 5%ethanolic Phosphomolybdic acid followed by charring. All intermediates were purified by flash column chromatography on silica gel (Merck Kieselgel 60) using the indicated mixtures of hexanes and ethyl acetate. Melting points were determined in open capillaries on a Thomas Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Laser Precision Analytical RFX-40 FTIR Spectrometer in the phase indicated. ^{1}H NMR and ^{13}C NMR were recorded on an IBM AF/250 spectrometer at 250 and 67.5 MHz respectively in $CDCl_3$ solutions unless otherwise indicated using the residual protiosolvent signal as internal reference. Mass spectra were obtained at The OhioState University' Chemical Instrumentation Center on either a VG 70-2505, a Nicolet FTMS-200 or a Finnigan MAT-900 mass spectrometer. Elemental Analysis were performed by Oneida Research Services, Inc. (Whitesboro, NY).

NOTE: All the synthetic compounds have been satisfactorily characterized by elemental analysis and/ or 1H NMR, 13C NMR, Mass spectroscopy. The analysis details have been excluded to maintain brevity of the report.

Synthesis of Estrogen A-ring fused heterocycles

General Procedure for synthesis of Methylenedioxy Estradiols:

Methylenedioxy estrone

A mixture of water(5.52mL), dibromomethane(2.92mL) and Adogen 464 (0.14 mL) was heated to reflux under Argon. A solution of catechol estrogen (200mg) and NaOH (2.7g) in water (13.8 mL) was added slowly such that the addition was complete in two hours. The mixture was continued to reflux for another hour. The reaction mixture was neutralized and extracted with chloroform, the organics were washed with water, dried ($MgSO_4$) and concenterated. The crude product was purified by flash chromatography (SiO_2/ 3% MeOH in CH_2Cl_2) to yield the methylenedioxy estrones in 65-70% yield.

($MgSO_4$) and concenterated. The crude product was purified by flash chromatography (SiO_2/ 3% MeOH in CH_2Cl_2) to yield the methylenedioxy estrones in 65-70% yield.

Methylenedioxy Estradiols
$NaBH_4$ (5mmol) was added in portions to a stirred solution of methylenedioxy estrone (0.5mmol) in dry MeOH at 0^0C. The resulting mixture was stirred for two hours with warming to room termperature. The reaction mixture was quenched with sat. NH4Cl and extracted with EtOAC, dried, concentratedand residue purified by column chromatography (SiO_2, Hexane/EtOAC 1:1) to yield the target compounds in 80% yield.

General Procedure for Synthesis of Dihydrobenzofuran and Dihydrobenzopyran estradiols

DEAD was added dropwise to a solution of hydroxyalkyl steroid (xmmol), PPh3 (xmmol) in THF under Argon. The resulting pale yellow solution was stirred under Argon at room temperature for 30 minutes. Reaction mixture was diluted with EtOAC and washed with water and brine, dried (MgSO4) and concentrated. Purified by column chromatography (SiO2, Hexane/EtOAC 2:1) to yield 85-95% of the cyclized products as colorless solids.

Please refer to Annual Report July' 97 for experimental details of synthesis of alkoxyalkyl estradiols and intermediates for synthesis of catechol estrogens.

Synthetic Approaches for Benzopyrone Combinatorial libraries

1,2-bisTBS Salicylic Acid
Imidazole (2.99g, 44mmol) was added to a solution of Salicylic Acid (1.38g, 10mmol) in DMF (12mL) and the resulting solution was stirred for 15 munutes under Argon after which TBSCl (3.3g, 22mmol) was added in a single portion. The mixture was stirred for 12 hrs and then DMAP (250mg) was added stirring continued till TLC indicated complete consumption of starting material. The mixture was diluted with water and extracted with EtOAc. Organics were washed with water, brine, dried (MgSO4) and concentrated. The crude product was purified by flash chromatography (SiO2, Hexane/ EtOAc 4:1) to yield 2.0g (56%) of the title compound.

General Procedure for Forming Acid chloride and coupling with terminal Alkyne
Oxalyl Chloride (1.2mmol) was added dropwise to a cold (0^0C) solution of 1,2-bisTBS Salicylic Acid (1.0mmol) and DMF (3drops) in freshly distilled dichloromethane (3ml). The solution was stirred at 00C for 2hours. The ice bath was removed and reaction was stirred for 18hours with warming to room temperature. The reaction mixture was concentrated on rotovap and the yield of the acid chloride was assumed to be quantitative. Et_3N (3ml) was added to the acid chloride followed by terminal alkyne (1.0mmol), CuI(1mg/ mmol alkyne) and $Pd(PPh_3)_2Cl_2$ (1mg/mmol alkyne). The reaction mixture was stirred for 12-15hrs under Agon. Upon completion of the reaction 10 mL of MeOH was added and reaction mixture concentrated on a rotovap. The residue was

dissolved in EtOAC and organics washed with water, brine, dried ($MgSO_4$), concentrated and purified (SiO_2, H/EA 5:1). The overall yields for the two steps range between 70-88% depending on the salicylic acid and terminal alkyne used.

Cyclization of Alkynones / Enaminones into Flavones:

Method A
To a solution of alkynone/ enaminone (0.21 mmol) and 18-crown-6 (0.52 mmol) in DMF (3ml) was added KF (0.42mmol) at 0^0C. The reaction mixture was stirred at ambient temperature for 4 hours. The reaction mixture was quenched with sat. NH_4Cl solution, extracted with EtOAC, washed with water, brine and purified (SiO_2 H/EA 4:1) to yield the cyclized flavones in 80% yields.(The cyclization yields range between 60-85%)

Method B
A solution of alkynone (0.2mmol) and PPTS (1mmol) in MeOH (2ml) was refluxed for 16 hours. The reaction mixture was extracted with EtOAC, washed with water, brine, dried (MgSO4) and concentrated to give the crude product which was purified by flash chromatography.

General procedure for addition of secondary amines to alkynones
A solution of alkynone (1mmol) in EtOH (5ml) was treated with secondary amine (diethylamine, diisopropyl amine, pyrrolidine, dimethylamine; 1.5 mmol) and the resulting solution was stirred under Argon for 6- 8 hours. The reaction mixture was concentrated and residue purified by column chromatography (SiO2, H/EA 4:1 to 2:1) to give the desired enaminones in quantative yields.

Conclusions

Potentiometric studies were carried out on both 2-HE_2 and 4-HE_2, each demonstrated quasi-reversibility at pH 7.4. The similarity in the degree of reversibility expressed as ΔE's and the similarity in formal potentials between these two compounds was somewhat surprising. There has been some debate over the differences in non-enzymatic activity and damaging effects between 2-HE_2 and 4-HE_2. For example, 2-HE_2 has been shown to form significant DNA strand breaks *in vitro* while 4-HE_2 failed to generate any strand breaks under the same conditions. In addition, 8-oxo-dG has been shown to increase in calf thymus DNA exposed to 4-HE_2 incubated with microsomal extracts from Syrian hamster livers but not with 2-HE_2.In agreement with the CV data, we have shown that 8-oxo-dG can be induced by both CE's, and that although 2-HE_2 was slightly more damaging than 4-HE_2 alone, the DNA damage was nearly equal when Cu(II) was present. Our studies indicate that there is a significant influence by copper on the DNA damaging potential of both 2-HE_2 and 4-HE_2 *in vitro,* which is possibly more significant than previously believed. The effect of LP on the HE_2's was shown to be protective in respect to 8-oxo-dG formation acting through a 2 e- oxidation pathway resulting in less ROS generation than that of the controls containing either 2-HE_2 or peroxide alone.

Potentiometric studies were also carried out on the HME_2's and ME_2's. These compounds are incapable of redox cycling and therefore would not be expected to cause significant formation of 8-oxo-dG. The lack of 8-oxo-dG formation by these analogs is in good agreement with the CV data. Spectroscopic studies indicated that the ME2's and HME2's were inert toward the LP catalyzed oxidation by peroxide.

Both the 2- and 4-HE_2's are capable of causing significant DNA damage at high micromolar concentrations while in the presence of loosely bound Cu(II) *in vitro*. However, the significance of oxidative DNA damage at physiologic concentrations of CE's *in vivo* would be difficult to predict. This is due in part to the ongoing debate over the exact concentration of total CE's that may accumulate and form locally in breast tissue. However the combined levels of CE's have not been shown to go over sub-nanomolar in serum. In addition, considerations for many cellular defenses such as conjugating enzymes, DNA binding proteins, and DNA repair have not been addressed in this paper. However, SOD, catalase and GSH all suppressed 8-oxo-dG formation significantly in the presence of 100 μM 2-OH-E_2 and 100 μM Cu(II)SO_4. Although LP was found to behave in a protective fashion, as mentioned earlier, there are reductases and peroxidases that have been shown to interact with the CE's through a 1 e- pathway generating the CE semiquinone and ROS. The ME_2's and HME_2's were shown to be stable analogs of the HE_2's and should prove to be useful in the study of HE_2's genotoxicity in cells. Extensions of this study to an ER positive breast cell model are underway.

In the Chemistry section novel Estrogen A-ring fused analogs have been synthesized. These analogs will be evaluated for their ability to inhibit estrogen hydroxylase enzymes. These studies can give us valuable information which will be used to design more potent analogs. Such compounds will be useful in further elucidating role of Catechol estrogens. We have optimized novel synthetic routes for making catechol estrogens. The methods described here are considerably superior over literature methods.

A series of alkoxyalkyl estradiols were evalated for their potential to inhibit tubulin polymerization. We have identified 2-Methoxymethyl estradiol as a more potent inhibitor of tubulin polymerization than 2-Methoxyestradiol. We are presently expanding on Structure Activity Relationships in this series of compounds. Efforts are underway to develop nonsteroidal agents as tubulin polymerization inhibitors.

The most significant achievement over the last year has been the successful initiation of project for design and synthesis of Benzopyrone Combinatorial Libraries. We have identified novel routes that seem well suited for making combinatorial libraries. Our method relies on mild reaction conditions, with ample scope to introduce diversity elements, and readily available building blocks. Reaction schemes have been developed which would enable us to use Resin-Capture techniques for generating the libraries. Additionally, it is possible to carry out the reactions on solid phase. Once generated the libraries would be screened for selective modulators of ERα, ERβ and inhibitors of tubulin polymerization inhibitors. Thus, synthesis and screening of benzopyrone libraries should we generate new lead molecules potentially useful in treatment and prevention of breast cancer.

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Synthesis and Biological Evaluation of 4-(Hydroxyalkyl)estradiols and Related Compounds

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A series of synthetic estrogens containing hydroxyalkyl side chains at the C-4 position of the A ring were designed as metabolically stable analogs of 4-hydroxyestradiol, a catechol estrogen. These synthetic steroids would facilitate investigations on the potential biological role of catechol estrogens and also enable further examination of the structural and electronic constraints on the A ring in the interaction of estrogens with the estrogen receptor. Catechol estrogens are implicated as possible causative agents in estrogen-induced tumorigenesis. 4-Hydroxyestradiol has weaker affinity for the estrogen receptor and exhibits lower estrogenic activity *in vivo*; on the other hand, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates. This report describes the synthesis and initial biochemical evaluation of 4-(hydroxyalkyl)estrogens and 4-(aminoalkyl)estradiols. The 4-(hydroxyalkyl)estrogens were prepared by oxidative hydroboration of 4-alkenylestradiols. The alkenylestradiols were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenylstannane. The (4-aminoalkyl)estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions. The substituted estradiols were evaluated for estrogen receptor binding activity in MCF-7 human mammary carcinoma cells, and 4-(hydroxymethyl)estradiol **1** exhibited the highest affinity with an apparent EC_{50} value of 364 nM. The relative activities for mRNA induction of the pS2 gene in MCF-7 cell cultures by the 4-(hydroxyalkyl)estrogens closely parallel the relative binding affinities. 4-(Hydroxymethyl)estradiol **1** did not stimulate the growth of MCF-7 cells at concentrations up to 1 μM. Thus, 4-(hydroxymethyl)estradiol **1** exhibited similar estrogen receptor affinity as the catechol estrogen, 4-hydroxyestradiol, and may prove useful in the examination of the biological effects of 4-hydroxyestrogens.

Introduction

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Currently, one out of nine American women will develop breast cancer in her lifetime. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, with these cancers characterized as containing estrogen receptors and requiring estrogen for tumor growth.[1] The possible biochemical roles of estrogens in the development of breast cancer remain to be fully elucidated.

Epidemiological studies have shown that women with breast cancer have higher estrogen levels than healthy control women and that estrogen levels are higher in populations characterized by high breast cancer rates.[2] An estimated 60–70% of human breast cancers are associated with sex hormone exposure. The fact that an early menarche and a late menopause are important risk factors for breast cancer suggests a role of the female sex hormones in the etiology of the disease.[3] Also, studies in experimental animals have shown estrogens to induce tumors in hormone-responsive tissues like mammary tissue, uterus, cervix, and pituitary.[4] Although estrogens have been implicated as carcinogens, the exact biochemical mechanisms by which estrogens may be tumorigenic remain to be established.

Catechol estrogens, oxidative metabolites of estrogens, have been suggested as possible causative agents in estrogen-induced tumorigenesis. Estrogens are converted to 2-hydroxy and 4-hydroxy derivatives by cytochrome P-450 hydroxylases.[5] Both 2-hydroxyestradiol and 4-hydroxyestradiol have weaker affinity for the estrogen receptor than estradiol and exhibit significantly lower estrogenic activity *in vivo*.[5] However, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones, and arene oxides.[6,7] These highly reactive moieties may be cytotoxic via reaction with proteins and nucleic acids.[8,9] Furthermore, the catechol estrogens have been shown to produce a variety of reactive oxygen species (ROS), such as the hydroxide, peroxide, and superoxide radicals.[10–12] These ROS have shown cytotoxic and genotoxic effects in several independent studies.[10,13,14]

Contrasting reports exist in the literature in regard to the tumorigenic potential of 2-hydroxyestradiols vs 4-hydroxyestradiols. Liehr *et al.* recently reported that microsomes prepared from human mammary adenocarcinoma and fibroadenoma have predominantly 4-hydroxylase activity, suggesting a mechanistic role of 4-hydroxyestradiol in tumor formation.[15] An earlier report demonstrated that 4-hydroxyestradiol formation is predominant in tissues susceptible to estrogen-induced tumorigenesis like Syrian hamster kidney and

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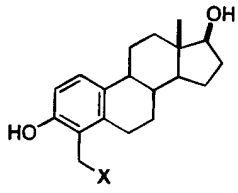
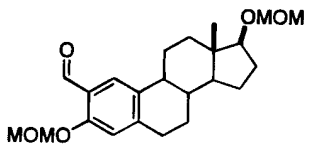
rat pituitary, whereas 2-hydroxyestradiol formation is predominant in rodent livers where tumors are not produced under similar conditions.[16-18] In contrast, Li and Trush found that 2-hydroxyestradiol produced oxidative damage and strand breaks of double-stranded DNA in the presence of micromolar concentrations of Cu(II), whereas 4-hydroxyestradiol failed to produce any DNA damage.[13,19]

In order to investigate the role of estrogen metabolites in tumor initiation and progression, we have designed, prepared, and reported on a series of 2-hydroxyalkyl derivatives.[20] The receptor binding and gene expression potential of these synthetic analogs closely parallels that of 2-hydroxyestradiol. Additionally, these compounds are not able to undergo oxidative metabolism at the 2-position. As a continuing part of this study, we have now prepared the corresponding 4-(hydroxyalkyl)estradiols **1**–**3**. These compounds were designed to provide 4-hydroxy-substituted estrogens that are not able to undergo further oxidative metabolism. On the other hand, compounds **1**–**3** do contain hydroxyl groups at the 3- and 4-positions that are available for hydrogen bonding during protein interactions with receptors and/or enzymes. The 4-(aminoalkyl)estrogens, compounds **4**–**6**, were also synthesized from the hydroxyalkyl derivatives to further elucidate electronic factors at the C-4 position that influence biological activity. Therefore, these analogs may prove useful as chemical probes for differentiating receptor-mediated vs redox-mediated events in estrogen-induced tumorigenesis. The synthesis and initial biochemical evaluation of these 4-hydroxyestradiol metabolite analogs are reported in this paper.



1: X=OH
2: X=CH_2OH
3: X=CH_2CH_2OH
4: X=NH_2
5: X=CH_2NH_2
6: X=$CH_2CH_2NH_2$



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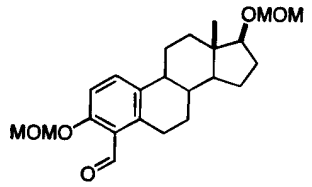
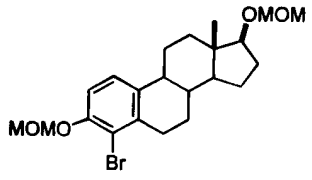


8



10

Results and Discussion

Chemistry. In our earlier work, the 2-(hydroxyalkyl)estradiols were prepared via homologation of a protected 2-formylestradiol **7**.[20,21] Pert and Ridley have previously demonstrated that the analogous 4-formylestradiol **8** could be prepared from **10** by lithium–halogen exchange and subsequent reaction of the organolithium with DMF.[22] Unlike the preparation of **7**, wherein yields in excess of 80% were routinely realized, only modest yields of **8** could be obtained. As this synthetic intermediate would be required in large quantities, the homologation of **8** was not considered to be the optimal route available for the preparation of **1**–**3**. Alternatively, the bisMOM-protected 4-bromoestradiol **10** was envisioned to be a suitable partner for a Stille cross-coupling reaction.[23] Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives, respectively, after hydroboration and oxidation.

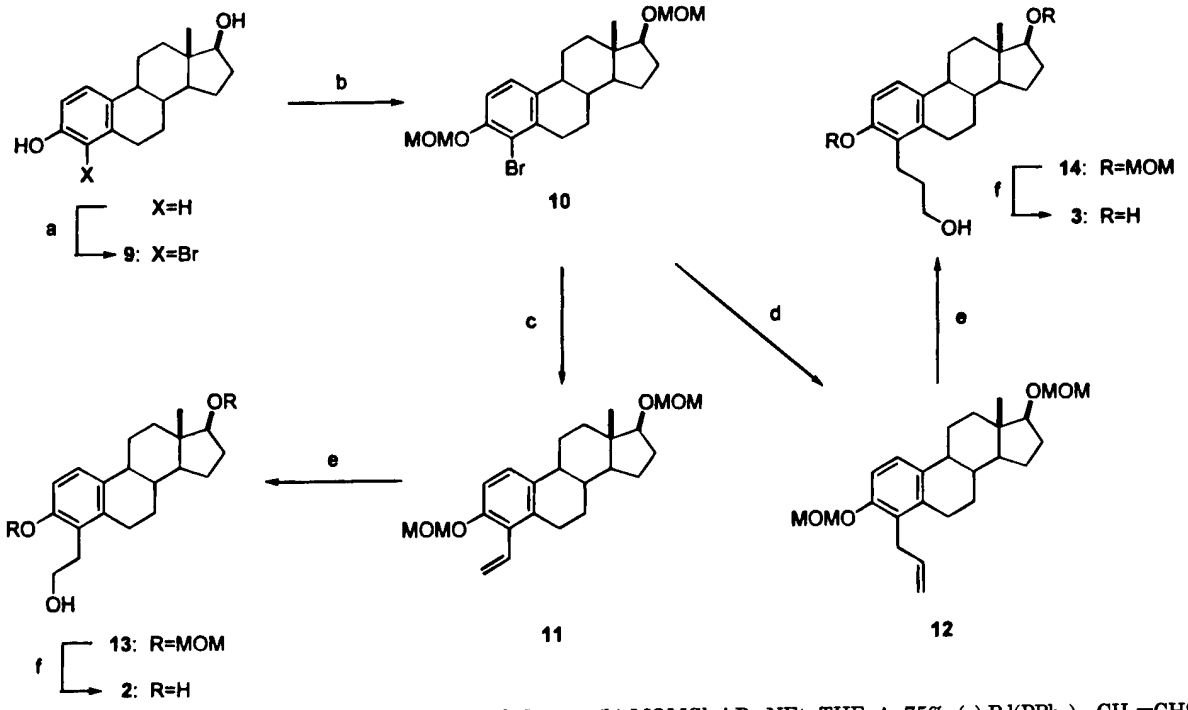
The synthesis commenced by brominating estradiol with *N*-bromosuccinimide in ethanol (Scheme 1), from which the required 4-bromoestradiol **9** precipitated and was obtained in 54% yield after recrystallization. The bromoestradiol was protected in 75% yield as its bis-MOM ether **10** with chloromethyl methyl ether, diisopropylethylamine in THF at reflux.[20] Using vinyltributyltin as the alkenyl donor, exploratory experiments were performed to determine the optimal reaction conditions required for the cross-coupling reaction. Thus, reaction of **10** with tetrakis(triphenylphosphine)-palladium(0) (0.06 molar equiv) and vinyltributyltin (2.1 molar equiv) in dry deoxygenated DMF afforded the desired 4-vinyl-bisMOM-estradiol **11** in 90% yield after heating at reflux overnight. Under similar reaction conditions, **10** was treated with allyltributyltin, affording 4-allyl-bisMOM-estradiol **12** in 94% yield. Using well-established chemistry, the unsaturated estradiols **11** and **12** were converted into alcohols **2** and **3**. Thus, hydroboration of **11** with BH_3·THF, followed by oxidative workup of the alkylborane with basic hydrogen peroxide, gave the desired alcohol **13**. The allylestradiol **12** was transformed into **14** in a similar fashion in 75% yield. Subsequent treatment of alcohols **13** or **14** with pyridinium *p*-toluenesulfonate (PPTS) gave the targeted triols in 70% and 61% yields, respectively.

An attempt was made to prepare the 4-formylestradiol **8** by way of a Stille-like reductive carbonylation as a prelude to preparing alcohol **1**. Treatment of **10** with carbon monoxide, tributyltin hydride, and tetrakis-(triphenylphosphine)palladium(0) in DMF at reflux failed to yield **8**. A control reaction in which **8**, prepared by the Pert and Ridley method, was heated for several hours in refluxing DMF demonstrated that it was thermally labile.[22] Indeed, a sample of **8** deteriorated simply on standing at room temperature for a few days.

In view of the instability of **8**, alternate routes for the preparation of other related derivatives were developed. Attempts have been made by Pert and Ridley to introduce an ester group by trapping the organolithium, generated from **10** and *n*-BuLi with alkyl chloroformates; these reactions were unsuccessful. Treatment of **10** with organolithium (*vide supra*) and carbon dioxide, followed by acidification and subsequent esterification with diazomethane, yielded the methyl ester **15** in 76% yield (Scheme 2). The MOM protecting groups were removed using PPTS in methanol at reflux in 88% yield. Subsequent reduction of the ester **16** with lithium aluminum hydride gave the benzyl alcohol **1** in 51% yield.

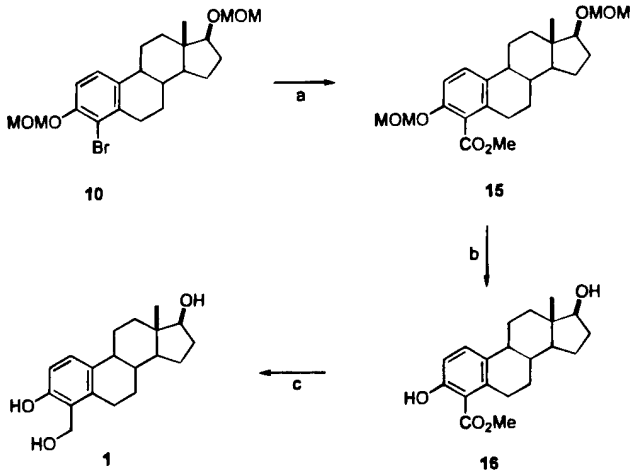
The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported.[20,21]

Scheme 1[a]



[a] Reagents and conditions: (a) *N*-bromosuccinimide, EtOH, 54%; (b) MOMCl, *i*-Pr_2NEt, THF, Δ, 75%; (c) $Pd(PPh_3)_4$, CH_2=$CHSnBu_3$, DMF, Δ, 90%; (d) $Pd(PPh_3)_4$, CH_3CH=$CHSnBu_3$, DMF, Δ, 94%; (e) (i) BH_3·THF, THF, 0 °C, (ii) NaOH, H_2O_2, Δ, **11** → **13** 39%, **12** → **14**, 82%; (f) PPTS, MeOH, Δ, **13** → **2** 80%, **14** → **3** 61%.

Scheme 2[a]



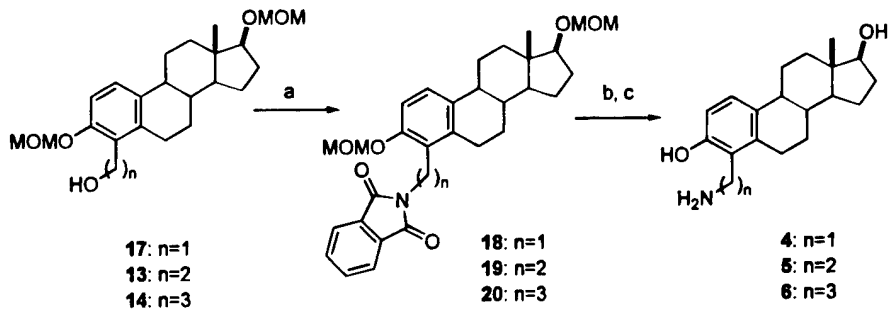
[a] Reagents and conditions: (a) (i) *n*-BuLi, THF, −78 °C, (ii) CO_2, −78 °C → rt; (iii) CH_2N_2, Et_2O, 0 °C, 76%; (b) PPTS, MeOH, Δ, 83%; (c) $LiAlH_4$, THF, 0 °C → rt, 51%.

Treatment of the bisMOM-protected 4-(hydroxylalkyl)-estradiols (**13**, **14**, **17**) with phthalimide under Mitsunobu conditions using triphenylphosphine (PPh_3) and diethyl azodicarboxylate (DEAD) yielded derivatives **18**–**20** in 70–80% yield (Scheme 3). Subsequent hydrazinolysis in refluxing ethanol gave the bisMOM-protected aminoestradiols which, upon treatment with methanolic HCl, gave the desired 4-(aminoalkyl)estradiols **4**–**6** in good yields.

Biology

The affinities of the synthetic 4-hydroxyestradiol analogs for the estrogen receptor were assessed in whole cell estrogen receptor binding assays using MCF-7 human mammary cancer cells.[20] The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations.[20] In addition, the cellular uptake and stability of analogs in the whole cell assay can be assessed. The EC_{50} value for estradiol binding to the estrogen receptor in these whole cell assays was found to be 0.180 nM (Table 1). The synthetic hydroxyestrogen analog with the highest estrogen receptor affinity was 4-(hydroxymethyl)estradiol **1**, exhibiting an EC_{50} value of 364 nM. Overall, the 4-substituted estradiol homologs exhibited significantly weaker affinity for the estrogen receptor than estradiol,

Scheme 3[a]



[a] Reagents and conditions: (a) PhthNH, DEAD, Ph_3P, THF; (b) NH_2NH_2, EtOH, Δ; (c) HCl, MeOH.

Table 1. Estrogen Receptor Affinity of 4-Substituted Estradiol Analogs

steroid	compd	EC_{50} (M)	log EC_{50} ± SD	RBA
estradiol		1.80×10^{-10}	−9.744 ± 0.102	100.00
4-hydroxyestradiol		5.06×10^{-7}	−6.295 ± 0.092	0.36
4-(hydroxymethyl)estradiol	**1**	3.64×10^{-7}	−6.438 ± 0.141	0.49
4-(hydroxyethyl)estradiol	**2**	6.20×10^{-7}	−6.207 ± 0.290	0.29
4-(hydroxypropyl)estradiol	**3**	3.32×10^{-6}	−5.479 ± 0.116	0.05
4-(aminomethyl)estradiol	**4**	NB[a]		
4-(aminoethyl)estradiol	**5**	2.50×10^{-6}	−5.600 ± 0.159	0.07
4-(aminopropyl)estradiol	**6**	NB		

[a] NB = no measurable binding of steroid at 10^{-5} M concentration.

Table 2. Induction of pS2 Gene Expression by 4-Substituted Estradiol Analogs

steroid	compd	EC_{50} (M)	log EC_{50} ± SD	% relative activity
estradiol		3.01×10^{-11}	−10.520 ± 0.217	100.00
4-hydroxyestradiol		6.54×10^{-8}	−7.184 ± 0.158	0.046
4-(hydroxymethyl)estradiol	**1**	1.17×10^{-8}	−7.933 ± 0.288	0.257
4-(hydroxyethyl)estradiol	**2**	1.48×10^{-7}	−6.829 ± 0.094	0.020
4-(hydroxypropyl)estradiol	**3**	2.95×10^{-6}	−5.530 ± 0.217	0.001

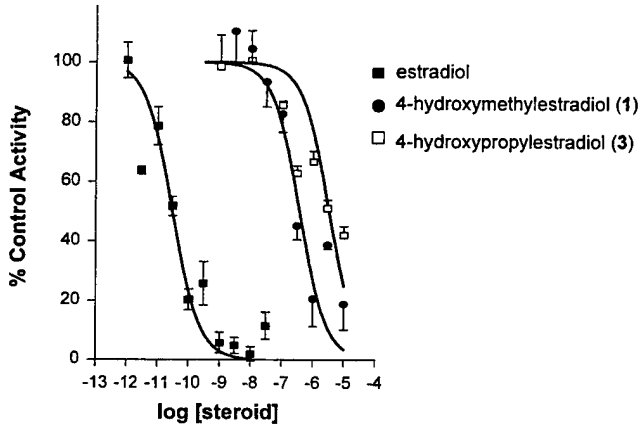


Figure 1. Estrogen receptor competitive binding assays for estradiol (■), 4-hydroxy-methylestradiol (●), and 4-hydroxypropylestradiol (□).

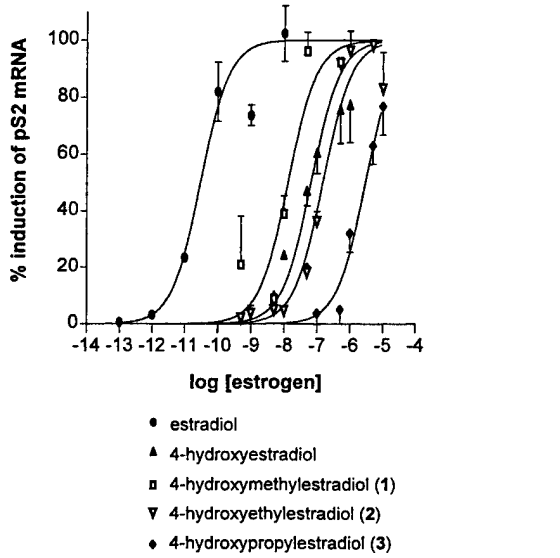


Figure 2. Induction of pS2 gene expression by estradiol (●), 4-hydroxyestradiol (▲), 4-(hydroxymethyl)estradiol (□), 4-(hydroxyethyl)estradiol (▽), and 4-(hydroxypropyl)estradiol (◆).

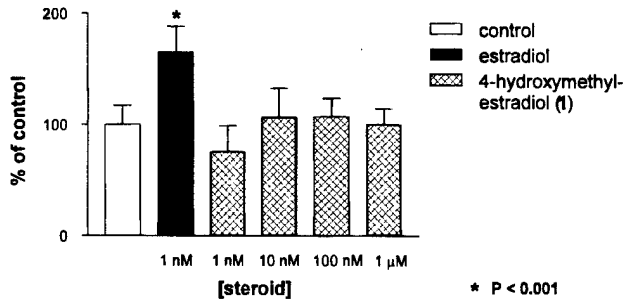


Figure 3. Comparison of mitogenic activities of estradiol (black bar), 4-(hydroxymethyl)estradiol (gray bar), and vehicle control (white bar) in MCF-7 human mammary carcinoma cell cultures.

with relative binding affinities (RBA; estradiol = 100) ranging from 0.49 for compound **1** to 0.05 for compound **3** (Table 1, Figure 1).

The relative estrogenic activities of the 4-hydroxyestradiol analogs were evaluated by examining the abilities of the synthetic compounds to induce estrogen-dependent gene expression in human breast cancer cells. In human MCF-7 mammary carcinoma cells, the induction of transcription of the pS2 gene is a primary response to estrogen.[24] The induction of pS2 mRNA expression by estradiol, 4-hydroxyestradiol, and 4-(hydroxyalkyl)estrogen analogs **1**−**3** was determined by RNA dot blot analysis. The EC_{50} value for estradiol induction of pS2 mRNA was found to be 0.030 nM. The estradiol homologs exhibited activity significantly weaker than that of estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) ranging from 0.257 for compound **1** to 0.001 for compound **3** (Table 2, Figure 2).

The effects of 4-(hydroxymethyl)estradiol **1** on the growth of hormone-dependent MCF-7 breast cancer cells was investigated since the compound exhibited the highest estrogen receptor affinity of the 4-hydroxylalkyl analogs synthesized. This mitogenic activity was determined by measuring [^{3}H]thymidine incorporation[25] at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 4-(Hydroxymethyl)estradiol did not affect cellular DNA synthesis in this breast cancer cell line, whereas estradiol at a concentration of 1 nM significantly increased MCF-7 cell growth (Figure 3).

Conclusions

The Stille cross-coupling and the carboxymethylation reaction reported here represent two efficient, previously unexplored synthetic routes for the functionalization of the 4-position of estradiol. The synthesis of the 4-(hydroxyalkyl)estrogens was accomplished by oxida-

tive hydroboration of 4-alkenylestradiols, which were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenylstannane. The 4-(aminoalkyl)estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions.

The substituted estradiols were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. The 4-(hydroxyalkyl)estradiols had significantly lower affinity for the estrogen receptor when compared with the endogenous ligand, estradiol. 4-(Hydroxymethyl)estradiol (**1**) exhibited the highest affinity of the synthetic compounds, with an apparent EC_{50} value of 364 nM, and it exhibited an affinity similar to that of the endogenous metabolite, 4-hydroxyestradiol, in the whole cell assays. On the other hand, the 4-(aminoalkyl)estradiols (**4**–**6**) exhibited either extremely weak or no affinity for the estrogen receptor.

Estradiol acts through the nuclear estrogen receptor to induce the transcription of a variety of hormone-responsive genes in target tissues, and induction of pS2 gene transcription is a primary response to estrogen observed in human MCF-7 mammary carcinoma cells.[22] The 4-(hydroxyalkyl)estradiols had significantly decreased efficacy for the induction of pS2 mRNA levels in MCF-7 cells when compared with the endogenous ligand, estradiol. Again, 4-(hydroxymethyl)estradiol (**1**) was the most potent among the synthetic compounds, with an apparent EC_{50} value of 11.7 nM. This synthetic compound was more effective than the endogenous metabolite, 4-hydroxyestradiol, which exhibited an apparent EC_{50} value of 65.4 nM.

Thus, the 4-(hydroxyalkyl)estradiols **1**–**3** exhibited both significantly weaker estrogen receptor affinities and abilities to induce pS2 gene expression in MCF-7 cell cultures. These results are consistent with the established structure–activity relationships of estrogens and the limitations of A ring substitutions on the estrogen molecule in producing estrogen receptor-mediated responses. On the other hand, 4-(hydroxymethyl)-estradiol (**1**) exhibited similar estrogen receptor affinity and similar induction of pS2 gene transcription as the catechol estrogen, 4-hydroxyestradiol. This catechol estradiol has been implicated as a possible causative agent in estrogen-induced tumorigenesis; however, *in vitro* and *in vivo* investigations with 4-hydroxyestradiol are difficult due to its chemical and biochemical instability. Thus, 4-(hydroxymethyl)estradiol (**1**) may be viewed as a chemically stable catechol estrogen homolog and may therefore prove useful in examination of the role of catechol estrogens in normal physiology and in pathological states, such as estrogen-induced tumorigenesis.

Experimental Methods

Synthesis: General Information. Estradiol was purchased from Steraloids (Wilton, NH). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. Amines were stirred over CaH_2, distilled, and then stored over KOH pellets. Silica gel TLC plates (60 F_{254}) were purchased from Analtech Inc. (Newark, NE) and visualized with a UV lamp and/or 5% ethanolic phosphomolybdic acid followed by charring. All intermediates were purified by flash column chromatography on silica gel (Merck Kieselgel 60) using the indicated mixtures of hexanes and ethyl acetate. Melting points were determined in open capillaries on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Laser Precision Analytical RFX-40 FTIR spectrometer in the phase indicated. ^1H NMR and ^{13}C NMR were recorded on an IBM AF/250 spectrometer at 250 and 67.5 MHz, respectively, in $CDCl_3$ solutions unless otherwise indicated using the residual protiosolvent signal as internal reference. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center on either a VG 70-2505, a Nicolet FTMS-200 or a Finnigan MAT-900 mass spectrometer. Elemental analyses were performed by Oneida Research Services, Inc. (Whitesboro, NY).

4-Bromoestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (10). MOMCl (5.7 mL, 75 mmol) was added dropwise to a cold (0 °C) solution of 4-bromoestradiol (5.26 g, 15.0 mmol) and diisopropylethylamine (21.3 mL, 89.3 mmol) in THF (125 mL). On completion of the addition, the reaction mixture was allowed to warm up to room temperature, stirred for 1 h at the same temperature, and then heated at reflux overnight. The mixture was allowed to cool, and then saturated NH_4Cl solution (100 mL) was added. The mixture was extracted with EtOAc (4 × 100 mL), and the combined organic solutions were washed with saturated aqueous brine (100 mL), dried ($MgSO_4$), and concentrated. The crude product was purified by flash column chromatography (SiO_2, hexane/ethyl acetate, 9:1) to afford a pale yellow solid, which was recrystallized from hexane to give 4.78 g (72%) of the desired compound as a colorless solid: mp 88–89 °C (lit. mp 97–98 °C); IR (KBr, cm^{-1}) 2925–2785, 1597, 1578, 1473, 1452, 1444, 1402, 1385, 1306, 1261, 1234, 1224, 1205, 1176, 1155, 1122, 1107, 1088, 980, 914, 897, 856; ^1H NMR 7.20 (1H, d, J = 8.6 Hz), 6.95 (1H, d, J = 8.6 Hz), 5.22 (2H, s), 4.69 (2H, AB q, J = 6.6 Hz, $\Delta\upsilon$ = 3.5 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.51 (3H, s), 3.37 (3H,s), 2.98 (1H, dd, J = 5.4, 17.9 Hz), 2.76–2.65 (1H, m), 2.31–2.11 (2H, m), 0.79 (3H, s); ^{13}C NMR 151.8, 137.8, 136.5, 124.8, 116.4, 113.5, 96.1, 95.4, 86.7, 56.2, 55.1, 50.1, 44.3, 43.0, 37.9, 37.3, 31.3, 28.2, 27.4, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 440.1341, obsd 440.1388.

4-Ethenylestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (11). A solution of **11** (440 mg, 1.0 mmol), vinyltributyltin (0.62 g, 2.0 mmol), and $Pd(PPh_3)_4$ (67 mg, 0.06 mmol) in DMF (15 mL) was deoxygenated by bubbling argon through it for 15 min. The solution was heated at reflux overnight, cooled to room temperature and diluted with ether (50 mL), washed with 5% NH_4OH (15 mL), water (4 × 20 mL), and brine (3 × 20 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (SiO_2, 4:1 hexane/ethyl acetate) to yield 344 mg (90%) of the title compound as a colorless oil, which solidified on standing to a colorless waxy solid: mp 55 °C; IR (KBr, cm^{-1}) 2930, 2888, 2847, 2023, 1698, 1586, 1476, 1444, 1158, 1112, 1055, 1045, 927; ^1H NMR 7.16 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.9 Hz), 6.40 (1H, dd, J = 11.7, 17.9 Hz), 5.55 (1H, dd, J = 2.3, 17.9 Hz), 5.52 (1H, dd, J = 2.3, 11.7 Hz), 5.12 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.36 (3H, s), 2.91–2.65 (2H, m), 0.74 (3H.s); ^{13}C NMR 152.9, 136.2, 134.4, 131.3, 127.1, 125.0, 119.7, 112.7, 96.1, 95.0, 86.7, 56.0, 55.1, 10.3, 44.5, 43.0, 38.0, 37.5, 28.23, 28.20, 27.4, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 386.2457, obsd 386.2443. Anal. ($C_{24}H_{34}O_4$) C, H.

4-(2′-Propenyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (12). A solution of **11** (1.50 g, 3.41 mmol), $Pd(PPh_3)_4$ (250 mg, 0.22 mmol) and allyl tri-*n*-butyl stannane (2.28 g, 6.90 mmol) in DMF (50 mL) was deoxygenated by bubbling argon through it for 15 min, and then the solution was heated at reflux overnight. After cooling, the solution was decanted off from the palladium, and the residual precipitated palladium was washed with ethyl acetate. The reaction solution was diluted with ethyl acetate, washed with water (3 × 50 mL), and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 10:1) to give 1.28 g (94%) of the desired allyl compound **14** as a colorless oil: IR (neat, cm^{-1}) 2931, 2850, 2825, 1637, 1481, 1446, 1254, 1225, 1205, 1190, 1151, 1134, 1105, 1082, 1055, 1028, 1007, 918; ^1H NMR 7.15

(1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.97–5.82 (1H, m), 5.17 (2H, s), 4.97 (1H, s), 4.92 (1H, dd, J = 1.5, 7.1 Hz), 4.65 (2H, AB q, J = 6.7, $\Delta\nu$ = 2.9 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.42 (2H, t, J = 6.6 Hz), 3.37 (3H, s), 2.91–2.66 (2H, m), 0.79 (3H, s); ^{13}C NMR 153.1, 136.5, 136.3, 134.3, 126.7, 124.1, 114.4, 111.7, 96.1, 94.8, 86.7, 55.9, 55.1, 50.3, 44.4, 43.0, 38.0, 37.5, 30.2, 28.2, 27.4, 26.7, 26.6, 23.1, 11.7; MS m/z (M^+) obsd 400.2613, calcd 400.2618. Anal. ($C_{25}H_{36}O_4$) C, H.

4-(2′-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol 3,-17β-Bis(methoxymethoxy) Ether (13). A solution of 1 M BH_3·THF (3.00 mL, 3.00 mmol) was added dropwise to a solution of **12** (286 mg, 0.75 mmol) in THF (6 mL) at 0 °C. On completion of the addition, the cooling bath was removed and the mixture stirred for 1 h; 1 M NaOH (3 mL) was added cautiously, and after the addition of 30% H_2O_2 (3 mL), the mixture was heated at reflux for 1 h. The mixture was allowed to cool, then ethyl acetate (75 mL) was added, and the organic solution was separated from the aqueous layer. The organics were washed with water (25 mL) and brine (25 mL), dried ($MgSO_4$), and concentrated. The residue was purified by MPLC (SiO_2, hexane/ethyl acetate, 2:1) to give 37 mg (12%) of a diastereomeric mixture of partially deprotected secondary alcohols, 92 mg (30%) of a mixture of two diastereomeric secondary alcohols, and 119 mg (39%) of the desired primary alcohol as a colorless oil, which crystallized on standing: mp 81–82 °C; IR (KBr, cm^{-1}) 3496, 3311, 2931, 2870, 2844, 2821, 1595, 1581, 1481, 1404, 1385, 1309, 1255, 1227, 1205, 1190, 1149, 1111, 1096, 1068, 1053, 1009, 912, 814; ^{1}H NMR 7.12 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, AB q, J = 6.6, $\Delta\nu$ = 3.3 Hz), 3.78 (2H, t, J = 7.0 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.46 (3H, s), 3.36 (3H, s), 2.97 (2H, t, J = 6.7 Hz), 2.96–2.88 (1H, m), 2.82–2.70 (1H, m), 0.79 (3H, s); ^{13}C NMR 153.6, 136.7, 134.6, 125.4, 124.4, 111.5, 96.1, 94.7, 86.7, 62.2, 56.0, 55.1, 50.3, 44.4, 43.0, 37.9, 37.4, 29.5, 28.2, 27.4, 27.1, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 404.2563, obsd 404.2574. Anal. ($C_{24}H_{36}O_5$) C, H.

4-(3′-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol 3,-17β-Bis(methoxymethoxy) Ether (14). A solution of 1 M BH_3·THF (12.4 mL, 12.4 mmol) was added dropwise to a solution of **14** (1.23 g, 3.08 mmol) in THF (25 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 1 h. NaOH (1 M, 25 mL) was added cautiously, then 30% H_2O_2 (25mL) was added, and the resulting mixture was heated at reflux for 1 h. The aqueous reaction mixture was extracted with ethyl acetate (3 × 100 mL), and the organic layer was washed with water (100 mL), brine (100 mL) and dried ($MgSO_4$), and concentrated. Column chromatography (SiO_2, hexane/ethyl acetate, 2:1) of the residue gave 1.06 g (82%) of the desired alcohol as a colorless oil: IR (neat, cm^{-1}) 3442, 2927, 1479, 1254, 1205, 1151, 1105, 1053, 1024, 920; ^{1}H NMR 7.13 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, AB q, J = 7.0, $\Delta\nu$ = 0 Hz), 3.67–3.37 (3H, m), 3.48 (3H, s), 3.37 (3H, m), 0.79 (3H, s), ^{13}C NMR 153.3, 136.2, 134.7, 128.6, 123.8, 111.5, 96.1, 95.0, 86.7, 62.4, 86.1, 55.1, 50.3, 44.4, 43.0, 38.0, 37.4, 32.0, 28.2, 27.4, 26.5, 23.1, 21.8, 11.7; MS m/z (M^+) calcd 418.2719, obsd 418.2712. Anal. ($C_{25}H_{38}O_5$) C, H.

4-(2′-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol (2). A solution of **13** (80 mg, 0.20 mmol) and pyridinium p-toluenesufonate (0.50 g, 2.00 mmol) in methanol (5 mL) was heated at reflux for 24 h. After the mixture as cooled to room temperature, ethyl acetate (50 mL) was added, and then the solution was washed with water (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was recrystallized from methanol and water to afford 50 mg (80%) of the alcohol: mp 229–230 °C; IR (KBr, cm^{-1}) 3338, 2966–2860, 1591, 1481, 1469, 1444, 1425, 1377, 1358, 1340, 1277, 1200, 1180, 1134. 1072, 1057, 1039, 1011, 818, 810; ^{1}H NMR (DMSO) 8.89 (1H, s), 6.92 (1H, d, J = 8.48 Hz), 6.56 (1H, d, J = 8.4 Hz), 4.62 (1H, br), 4.47 (1H, d, J = 4.8 Hz), 3.54–3.41 (1H, m), 2.84–2.59 (4H, m), 0.63 (3H, s); ^{13}C NMR 151.9, 135.5, 130.7, 123.2, 122.7, 112.1, 79.9, 59.9, 49.5, 43.7, 42.5, 37.8, 36.5, 29.8, 29.4, 27.0, 26.1, 22.6, 11.0; (M^+) calcd 316.2038, obsd 316.2032. Anal. ($C_{20}H_{28}O_3$·0.5H_2O) C, H.

4-(3′-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol (3). A solution of the alcohol **14** (173 mg, 0.41 mmol) and pyridinium p-toluenesulfonate (0.50 g, 2.0 mmol) in methanol (5 mL) was heated at reflux for 24 h. After cooling, the reaction mixture was diluted with ethyl acetate (50 mL), and then it was washed with water (3 × 25 mL) and brine (25 mL), dried, and concentrated. The residue was recrystallized from methanol/water to give 61 mg (61%) of the desired alcohol as a colorless solid: mp 240–242 °C; IR (KBr, cm^{-1}) 3392, 3249, 2971, 2916, 2864, 1591, 1491, 1471, 1446, 1425, 1379, 1362, 1280, 1080, 1059, 1034, 1003, 814, 808; ^{1}H NMR (DMSO) 10.28 (1H, s), 6.90 (1H, d, J = 8.5 Hz), 6.56 (1H, d, 8.5 Hz), 4.47 (1H, d, J = 4.8 Hz), 4.41 (1H, t, J = 5.2 Hz), 3.54–3.36 (2H, m), 2.80–2.45 (5H, m), 2.22–2.12 (1H, m), 2.09–2.00 (1H, m), 1.84–1.80 (3H, m), 1.59–1.43 (3H, m), 1.39–1.04 (7H, m), 0.54 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 125.9, 122.8, 112.1, 79.9, 60.9, 49.6, 43.8, 42.6, 37.9, 36.5, 31.8, 27.0, 26.2, 25.9, 22.6, 21.8, 11.1; MS m/z (M^+) calcd 330.2195, obsd 330.2192. Anal. ($C_{21}H_{30}O_3$·0.25H_2O) C, H.

4-Carboxy-3,17β-Bis(methoxymethoxy)estra-1,3,5(10)-triene-3,17β-diol Methyl Ester (15). n-BuLi (3.5 mL, 5.65 mmol) was added dropwise to a solution of **10** (1.15 g, 2.61 mmol) in THF (50 mL) at −78 °C. After 1 h of stirring at this temperature, several pieces of dry ice were added, and then the cooling bath was removed. After being warmed to room temperature, the reaction mixture was diluted with ether (50 mL) and then extracted with 5% KOH (5 × 50 mL). The combined basic extracts were acidified to pH 5 with concentrated HCl and then extracted with ether (6 × 50 mL). The combined ethereal extracts were washed with brine (50 mL), dried ($MgSO_4$), and concentrated to give the crude acid. The acid was suspended in ether (20 mL), and an ethereal solution of diazomethane was added to it at 0 °C. After 30 min, sufficient acetic acid was added to the reaction mixture to discharge the yellow coloration. The reaction mixture was washed with $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), concentrated, and purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to give 0.82 g (76%) of the desired product as a colorless oil, which crystallized after a few days of standing at room temperature: mp 66–68 °C; IR (KBr, cm^{-1}) 2924, 2862, 1728, 1585, 1481, 1440, 1384, 1273, 1257, 1248, 1155, 1126, 1103, 1061, 1049, 1031, 793; ^{1}H NMR 7.25 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.13 (2H, s), 4.64 (2H, AB q, J = 7.0, $\Delta\nu$ = 0 Hz), 3.88 (3H, s), 3.59 (1H, t, J = 8.4 Hz), 3.44 (3H, s), 3.36 (3H, s), 2.79–2.73 (2H, m), 2.29–1.99 (4H, m), 1.95–1.83 (1H, m), 0.79 (3H, s); ^{13}C NMR 168.9, 151.6, 134.7, 134.6, 127.4, 124.8, 112.6, 96.2, 95.0, 86.7, 56.0, 55.1, 51.9, 50.1, 44.1, 43.0, 38.1, 37.3, 28.2, 26.8, 26.6, 26.4, 23.1, 11.7; MS m/z (M^+) calcd 418.2355, found 418.2337. Anal. ($C_{24}H_{34}O_6$) C, H.

4-Carboxy-3,17β-estra-1,3,5(10)-triene-3,17β-diol Methyl Ester (16). A solution of the ester **15** (0.44 g, 1.10 mmol) and pyridinium p-toluenesulfonate (2.77 g, 11.0 mmol) in MeOH (10 mL) was heated to reflux for 36 h. After cooling and addition of EtOAc (100 mL), the organic solution was washed with water (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was filtered through a short pad of silica gel (ethyl acetate/hexane, 1:2) to give 0.30 g (83%) of **16** as a colorless oil, which crystallized on standing: mp 135–136 °C (MeOH/H_2O); IR (KBr, cm^{-1}) 3435, 2920, 2866, 1718, 1591, 1446, 1427, 1383, 1361, 1344, 1288, 1267, 1230, 1217, 1190, 1171, 1136, 1122, 1059, 1039, 1011, 960; ^{1}H NMR 10.72 (1H, s), 7.38 (1H, d, J = 8.8 Hz), 6.80 (1H, d, J = 8.8 Hz), 3.93 (3H, s), 3.72 (1H, t, J = 8.4 Hz), 3.10–3.04 (2H, m), 2.29–2.06 (3H, m), 1.97–1.84 (2H, m), 0.77 (3H, s); ^{13}C NMR 172.0, 160.0, 139.3, 132.4, 132.1, 115.2, 112.7, 81.8, 51.9, 501, 44.7, 43.3, 37.9, 36.9, 30.7, 29.8, 27.3, 26.9, 23.0, 11.1; MS m/z (M^+) calcd 330.1831, found 330.1835. Anal. ($C_{20}H_{26}O_4$·0.5H_2O) C, H.

4-Hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (1). $LiAlH_4$ (70 mg, 1.89 mmol) was added portionwise to a solution of the ester **16** (51 mg, 0.15 mmol) in THF (5 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 4 h. When the reaction was complete, water (0.07 mL), 15% NaOH (0.07 mL), and water (0.21 mL) were added. Once a granular precipitate had formed, it was removed by suction filtration through Celite, washed with MeOH, and concentrated to yield 23 mg

(51%) of the desired alcohol as a colorless solid: mp > 270 °C; IR (KBr, cm^{-1}) 3375, 3240, 2960, 2931, 2920, 2866, 2850, 1591, 1479, 1448, 1429, 1383, 1352, 1286, 1252, 1078, 1065, 1009, 820 ^1H NMR 9.01 (1H, s), 6.99 (1H, d, J = 8.5 Hz), 6.58 (1H, d, J = 8.5 Hz), 4.53 (1H, m), 4.48 (2H, AB q, J = 4.8, $\Delta\nu$ = 7.7 Hz), 3.55–3.50 (1H, m), 2.98–2.80 (1H, m), 2.78–2.63 (1H, m), 2.30–2.05 (1H, m), 2.03–1.86 (1H, m), 1.85–1.63 (3H, m), 0.67 (3H, s); ^{13}C NMR 153.1, 136.3, 130.7, 124.7, 124.4, 112.7, 79.9, 54.7, 49.5, 43.7, 42.6, 37.9, 36.5, 29.8, 26.8, 26.2, 25.4, 22.6, 11.0; MS m/z (M^+) calcd 302.1875, obsd 302.1883. Anal. ($C_{19}H_{26}O_3 \cdot 0.25H_2O$) C, H.

4-(Hydroxymethyl)-3,17β-bis(methoxymethoxy)estra-1,3,5(10)-triene (17). $LiAlH_4$ (340 mg, 9.19 mmol) was added portionwise to a solution of **15** (0.50 g, 1.20 mmol) in THF (30 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature over 1 h and then stirred for 4 h. Water (0.34 mL), 15% NaOH (0.34 mL), and water (1.00 mL) were added successively, and then the resulting grannular precipitate was removed by filtration through a pad of Celite and $MgSO_4$ (1:1). After concentration of the filtrate it was chromatographed to give 0.43 g (90%) of the desired alcohol as a colorless oil, which slowly crystallized: mp 80–81 °C; IR (KBr, cm^{-1}) 3479, 2964–2814, 1597, 1583, 1481, 1441, 1400, 1385, 1255, 1242, 1228, 1153, 1107, 1092, 1063, 1045, 1031, 1005, 995, 955, 903; ^1H NMR 7.23 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.7 Hz), 5.19 (2H, AB q, J = 6.7, $\Delta\nu$ = 3.4 Hz), 4.74 (2H, m), 4.64 (2H, AB q, J = 6.6, $\Delta\nu$ = 3.4 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.48 (3H, s), 3.36 (3H, s), 3.10–2.92 (1H, m), 2.90–2.81 (1H, m), 2.33–1.91 (6H, m), 1.76–1.11 (9H, m), 0.79 (3H, m); ^{13}C NMR 153.9, 136.8, 135.1, 128.0, 126.2, 112.6, 96.1, 95.5, 86.7, 56.7, 56.3, 55.1, 50.2, 44.4, 43.0, 37.9, 37.4, 28.2, 27.2, 26.6, 26.5, 23.1, 11.7; MS m/z (M^+) calcd 390.2397, obsd 390.2391. Anal. ($C_{23}H_{34}O_5$) C, H.

Phthalimides 23–24: General Procedure. DEAD (0.42 mL, 2.40 mmol) was added dropwise to a solution of the alcohol **13**, **14**, or **17** (0.80 mmol), phthalimide (0.35 g, 2.40 mmol), and triphenylphosphine (0.63 g, 2.40 mmol) in THF (10 mL) at room temperature and then stirred overnight. The solvent was removed *in vacuo*, and then the residue was dissolved in EtOAc (100 mL), washed with 5% aqueous KOH (4 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to afford the substituted phthalimides **18–20** as colorless or pale yellow oils, which solidified on standing.

4-(Phthalimidoylmethyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (18): 79%; mp 104–106 °C; IR (KBr, cm^{-1}) 2951, 2925, 2868, 1711, 1479, 1396, 1385, 1348, 1255, 1151, 1113, 1092, 1055, 1028, 1009, 993, 962, 949, 918, 723; ^1H NMR 7.79–7.74 (2H, m), 7.69–7.64 (2H, m), 7.20 (1H, d, J = 8.7 Hz), 6.90 (1H, d, J = 8.7 Hz), 5.12 (2H, s), 4.90 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.36 (3H, s), 3.31 (3H, s), 3.20–3.13 (1H, m), 2.97–2.86 (1H, m), 2.29–1.94 (3H, m), 0.78 (3H, s); ^{13}C NMR 167.9, 137.6, 134.2, 133.7, 132.3, 126.0, 123.0, 122.0, 111.4, 96.1, 94.6, 86.7, 55.8, 55.1, 50.3, 44.4, 43.0, 37.8, 37.4, 34.3, 28.2, 27.4, 26.9, 26.5, 23.1, 11.7; MS m/z (M^+) calcd 519.2621, obsd 519.2634. Anal. ($C_{32}H_{39}NO_6$) C, H, N.

4-(Phthalimidoylethyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (19): 87%; mp 117–120 °C; IR (KBr, cm^{-1}) 2930, 2880, 1772, 1716, 1505, 1430, 1393, 1360, 1152, 1119, 1109, 1070, 1052, 998, 900, 720; ^1H NMR 7.83–7.67 (4H, m), 7.15 (1H, d, J = 8.68 Hz), 6.90 (1H, d, J = 8.67 Hz), 5.16 (2H, s), 4.64 (2H, AB q, J = 6.8, $\Delta\nu$ = 2.9 Hz), 3.81 (1H, t, J = 8.2 Hz), 3.48 (3H, s), 3.35 (3H, s), 3.03–2.75 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.1, 153.6, 136.6, 134.4, 133.7, 132.3, 124.9, 124.6, 123.0, 111.2, 96.0, 94.5, 86.6, 56.0, 50.1, 44.3, 42.9, 37.8, 37.3, 36.8, 28.1, 27.3, 26.7, 26.5, 25.3, 23.0, 11.7; MS m/z (M^+) calcd 533.2767, obsd 533.2772 Anal. ($C_{31}H_{37}NO_6$) C, H, N.

4-(Phthalimidoylpropyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (25): 93%; mp 134–135 °C; IR (KBr, cm^{-1}) 2955, 2935, 2894, 2786, 1777, 1726, 1485, 1478, 1445, 1394, 1363, 1153, 1122, 1086, 1055, 1040, 922, 720; ^1H NMR 7.85–7.80 (2H, m), 7.73–7.67 (2H, m), 7.13 (1H, d, J = 8.7 Hz), 6.87 (1H, d, J = 8.7 Hz), 5.09 (2H, s), 4.64 (2H, AB q, J = 6.9, $\Delta\nu$ = 1.7 Hz), 3.78 (2H, t, J = 7.2 Hz), 3.59 (1H, t, J = 8.3 Hz), 3.39 (3H, s), 3.36 (3H, s), 2.88–2.64 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.3, 153.0, 135.7, 134.1, 133.7, 132.2, 128.2, 123.7, 123.0, 111.2, 96.0, 94.4, 86.7, 55.8, 55.0, 50.2, 44.3, 42.9, 38.4, 37.8, 37.4, 28.1, 27.4, 26.6, 26.5, 23.3, 23.0, 11.6; MS m/z (M^+) calcd 547.1693, obsd 547.2908. Anal. ($C_{33}H_{41}NO_6$) C, H, N.

Amines 4–6: General Procedure: A solution of the phthalimides **18–20** (0.46 mmol) and hydrazine (1 mL) in ethanol (10 mL) was heated at reflux for 1 h. After cooling, ethyl acetate (50 mL) was added, and then the mixture was washed with 5% KOH solution (3 × 25 mL) and brine (25 mL), dried ($MgSO_4$), and concentrated. The residue was dissolved in methanol (5 mL) and cooled to 0 °C, and then HCl was bubbled through it for 15 min. The cooling bath was removed, and then stirring was continued for 3 h. The reaction mixture was concentrated and then redissolved in methanol, and the resulting green solution was decolorized with charcoal. After filtration and concentration, the residue was taken up in the minimum amount of methanol, and the product was precipitated out by the addition of ether, affording the amine salts as colorless or yellow solids.

4-(Aminomethyl)estra-1,3,5(10)-triene-3,17β-diol (4): 90%; mp > 270 °C; IR (KBr, cm^{-1}) 3444–2868, 1620, 1591, 1509, 1491, 1473, 1450, 1379, 1352, 1323, 1284, 1261, 1219, 1201, 1188, 1080, 1057, 1007, 945, 814; ^1H NMR (DMSO) 8.42 (4H, brs), 7.15 (1H, d, J = 8.6 Hz), 6.75 (1H, d, J = 8.6 Hz), 4.48 (1H, brs), 3.89 (1H, s), 3.52 (1H, *t*, J = 8.2 Hz), 2.94–2.70 (2H, m), 2.30–2.22 (1H, m), 1.95–1.70 (3H, m), 1.65–1.49), 0.64 (3H, s); ^{13}C NMR 152.9, 135.4, 130.1, 125.5, 116.8, 111.5, 78.8, 48.3, 42.6, 41.5, 36.7, 35.4, 32.9, 28.8, 25.6, 25.2, 24.8, 21.6, 10.0; MS m/z (M^+ − HCl) calcd 301.2037, obsd 301.2042. Anal. ($C_{19}H_{28}NO_2Cl \cdot H_2O$) C, H, N.

4-(Aminoethyl)estra-1,3,5(10)-triene 3,17β-diol (5): 87%; mp >270 °C; IR (KBr, cm^{-1}) 3355, 3299, 3059, 2865, 1589, 1471, 1447, 1383, 1362, 1281, 1270, 1142, 1086, 1066, 1020, 943, 809; ^1H NMR (DMSO) 8.5 (4H, brs), 6.92 (1H, d, J = 8.12 Hz), 6.54 (1H, d, J = 8.03 Hz), 4.49 (1H, brs), 3.52 (1H, *t*, J = 8.01 Hz), 2.80–2.55 (4H, m), 0.64 (3H, s); ^{13}C NMR 153.7, 134.9, 131.2, 123.1, 113.0, 79.8, 49.7, 44.9, 42.4, 38.1, 36.2, 29.9, 27.0, 26.3, 26.1, 22.9, 11.3; MS m/z (M^+ − HCl) calcd 315.2198, obsd 315.2201.

4-(Aminopropyl)estra-1,3,5(10)-triene-3,17β-diol (6): 86%; mp >270 °C; IR (KBr, cm^{-1}) 3362, 3276, 3056, 3022, 2962, 2925, 2863, 1635, 1589, 1489, 1443, 1280, 1208, 1133, 1058, 813; ^1H NMR (DMSO) 9.2 (1H, brs), 7.91 (3H, s), 6.94 (1H, d, J = 8.49 Hz), 6.62 (1H, d, J = 8.38 Hz), 4.50 (1H, d, J = 4.6 Hz), 3.51–3.44 (3H, m), 2.78–2.73 (4H, m), 0.63 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 124.4, 123.2, 112.2, 79.8, 49.5, 43.7, 42.5, 40.5, 37.8, 36.4, 29.8, 26.9, 26.3, 26.1, 25.8, 22.6, 22.0, 11.0; MS m/z (M^+ − HCl) calcd 329.2355, obsd 329.2354.

Biological Evaluations. General Information. [2,4,6,7-^3H]Estradiol (98.4 Ci/mmol, ^3H-E_2) was purchased from Dupont/NEN (Boston, MA) and was used as received. MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and cells were incubated in a humidified CO_2 incubator (Forma model 3052) with 5% CO_2 atmosphere. A modified Eagle's minimum essential medium (MEM) supplemented with essential amino acids (1.5×), vitamins (1.5×), nonessential amino acids (2×), and L-glutamine (1×) was obtained from Gibco BRL (Long Island, NY) and was used for maintaining the cells. The sterilized liquid medium was prepared by the OSU Comprehensive Cancer Center by dissolving the powder in water containing sodium chloride (0.487 g/L), pyruvic acid (0.11 g/L), and sodium bicarbonate (1.5 g/L) and the pH adjusted to 6.8. Fetal calf serum was obtained from Gibco BRL. Steroids were removed from heat-inactivated fetal calf serum by two treatments with dextran-coated charcoal at 57 °C. Tissue culture flasks and supplies were obtained from Corning Glass Works (Corning, NY). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 963 (Dupont/NEN) as the counting solution. Probes for RNA dot blot analysis (pS2:ATCC 57137; 36B4:ATCC 65917) were obtained as purified plasmids from the American Type Culture Collection and amplified by PCR for use in hybridization. Primers used were synthesized by OLIGOS, ETC (Wilsonville, OR) and were as follows:

For pS2:	sense	5'-ATC CCT GAC TCG GGG TCG CCT TTG-3'
	antisense	5'-CAA TCT GTG TTG TGA GCC GAG GCA CAG-3'
For 36B4:	sense	5'-AAA CTG CTG CCT CAT ATC CG-3'
	antisense	5'-TTT CAG CAA GTG GGA AGG TG-3'

Probes were labeled by random priming with Klenow fragment. Analysis of the RNA dot blots were performed on a Molecular Dynamics PhosphoImager SI.

Whole Cell Estrogen Receptor Studies.[20] MCF-7 cells were maintained in a similar fashion as described above. Cells from 90−100% confluent cultures were harvested by treatment with 0.01% trypsin solution, and the washed cell pellet was divided into 9.4 cm^2 wells on a six-well plate at $(1.5-2) \times 10^5$ cells/well in modified MEM (2−3 mL) containing 10% steroid free fetal calf serum and gentamycin (20 mg/mL). After 12−24 h at 37 °C, the culture media was removed and replaced and with serum-free modified MEM media (888 μL) containing insulin (5.0 mg/L), transferrin (5.0 mg/L), glutamine (2 mM), and albumin (2.0 mg/mL). After 48 h, the media was removed, fresh serum-free-modified MEM media added, and the synthetic estrogens **1**−**6** at various concentrations (3×10^{-5} to 1×10^{-5} M, 100 μL) were added and incubated for 10 min at 37 °C. To determine total binding, [^{3}H]estradiol (3.0 nM, 1.0 μCi) was added, and the plates were then incubated for 1 h at 37 °C. The cells were washed twice with PBS at 4 °C and then 95% ethanol (1 mL) was added, followed by standing for 30 min at room temperature. An aliquot (500 μL) of the ethanol solution was added to Formula 963 and counted on a liquid scintillation counter. The blank samples with no cells and nonspecific binding samples, containing 6 μM unlabeled estradiol, were performed in a comparable manner. Specific binding of [^{3}H]estradiol was calculated by subtracting the nonspecific binding data from total binding data. The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [^{3}H]estradiol binding and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

pS2 Induction. MCF-7 cells were maintained in a similar fashion as described above. Cells were plated at a concentration of 5.5×10^5 cells/25 cm^2 flask. After 2 days of growth, the cells were rinsed with Ca^{2+}, Mg^{2+} free PBS and placed on defined media for 48 h. Defined media contained DMEM/F12 media (Gibco BRL) supplemented with human albumin (2.0 mg/ml), transferrin (5.0 mg/L), bovine insulin (5.0 mg/L), and *l*-glutamine (2 mM). After addition of fresh defined media, the cells were dosed with compound (10^{-10}−10^{-5} M), 10 nM 17β-estradiol (Sigma, St. Louis, MO), or carrier (95% ethanol). Each compound was tested in triplicate. After 24 h, total cellular RNA was isolated by an adaptation of the method of Chomczynski and Sacchi.[20] The cells were lysed with a 4 M guanidine isothiocyanate solution, and the lysate was acidified with 3 M sodium acetate, pH 5.2 (1:10 vol). After addition of 3 M NaOAc, pH 5.2 (1:10 vol), RNA was extracted twice using water-saturated phenol:chloroform:isoamyl alcohol (60:24:1) at pH 4.0. A final extraction using an equal volume of chloroform:isoamyl alcohol (25:1) was performed. RNA from the resulting aqueous layer was precipitated with an equal volume of 2-propanol at −20 °C for 1 h. The RNA was pelleted at 15000*g* for 30 min at 4 °C. The resulting pellet was washed twice with 70% ethanol and once with 95% ethanol. Dried pellets were resuspended in 30 *u*L of Dnase-, Rnase-free molecular biology grade water (Sigma Chemical Co.). Quantification of RNA in each sample was performed using the absorbance at 260 nm.

Dot Blot Analysis. A denaturing solution containing 50% formamide, 7% formaldehyde, and 1× SSPE was added to 15 μg of RNA from each sample. The RNA was denatured at 68 °C for 15 min. Two volumes of 10× SSPE was added to each sample. The samples were loaded onto a 0.45 μm, positively charged, nylon membrane (Schleicher and Schuell, Keene, NH) using gentle suction through a 96-well dot blot manifold (BioRad, Hercules, CA). Membranes probed for pS2 gene expression were loaded with 10 μg RNA, the remaining 5 μg was loaded onto a membrane probed for the control gene, 36B4. Membranes were baked at 80 °C for 1 h and then incubated for at least 3 h in a prehydridization solution containing 5× SSPE, 5× Denhardts Reagent, 2% SDS, 100 μg/mL salmon sperm DNA, and 50% formamide. pS2 and 36B4 cDNA was prepared as described above and used to make ^{32}P-radiolabeled probes using random primers in the RadPrime Kit (Gibco BRL). Probes with specific activity ranging from 5.0×10^5 to 2.0×10^6 cpm/ng were used. Membranes, probed separately for pS2 or 36B4, were incubated for 48 h or 24 h, respectively, in hybridization solution containing 5× SSPE, 5× Denhardts reagent, 1% SDS, 100 μg/mL salmon sperm DNA, 10% PEG, and 50% formamide. The membranes were washed in 0.5× SSPE, 60′, at 55 °C; 0.1× SSPE, 60′, 60 °C; and 0.1× SSPE, 60′, 65 °C. Phosphor screens were exposed for at least 1 h and scanned on the PhosphorImager SI (Molecular Dynamics). Quantification of the signal was performed using ImageQuaNT software (Molecular Dynamics). The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal induction of pS2 mRNA and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

Cell Growth Assay. Human mammary carcinoma cell lines were maintained in 75-cm^2 plastic flasks at 37 °C in a modified Eagle's MEM (10 mL) containing 10% fetal calf serum and gentamycin (20 mg/L). For cell growth determinations, the mammary carcinoma cells were divided into 9.4 cm^2 wells at approximately 100 000 cells/well in modified MEM (2 mL) containing 10% steroid-free fetal calf serum and gentamycin (20 mg/l). After 2 days, media was changed to serum-free-modified MEM and experiments initiated. To determine dose-dependent effects, varying concentrations of 4-(hydroxymethyl)-estradiol **1** (3 nM to 10 μM in 5 μL of 95% ethanol) were added and incubated for 4 days. Effects on cell division were measured by the addition of [^{3}H]thymidine (1 μCi/well), followed by incubation for 2 h, cell lysis, and determination of [^{3}H]thymidine incorporation into DNA. Each experiment was carried out in quadriplicates, and test compounds were evaluated in experiments performed at least three different times. Statistical differences between control and treated groups were determined using the Student's *t* test.

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